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DRUG-DIAGNOSTICS CO-DEVELOPMENT IN ONCOLOGY

Topic Editor
Jan Trøst Jørgensen

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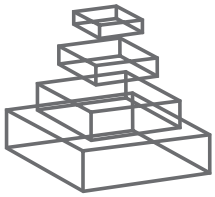

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ISSN 1664-8714
ISBN 978-2-88919-332-5
DOI 10.3389/978-2-88919-332-5

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DRUG-DIAGNOSTICS CO-DEVELOPMENT IN ONCOLOGY

Topic Editor:
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The idea of combining drugs and diagnostics in oncology is not new. When the selective estrogen receptor modulator tamoxifen was developed in the 1970's for the treatment of breast cancer a positive correlation between receptor status and treatment outcome was found. As a result of this research, it was suggested to use the estrogen-receptor assay as a diagnostic test for selection of patients for tamoxifen treatment. Despite this suggestion was put forward nearly 40 years ago the adaptation of the drug-diagnostic co-development model has been relatively slow and it is only within the last decade that it has gained more widespread acceptance. The parallel development of the monoclonal antibody trastuzumab (Herceptin®, Roche/Genentech) and the immunohistochemistry assay for HER2 protein overexpression (HercepTest™, Dako) seems to have served as an inspiration to a number of stakeholders such as pharma and diagnostic companies, regulatory agencies, and academia. In recent years we have seen an increasing number of oncology drug development projects that have taken advantage of the drug-diagnostic co-development model, as outline below.

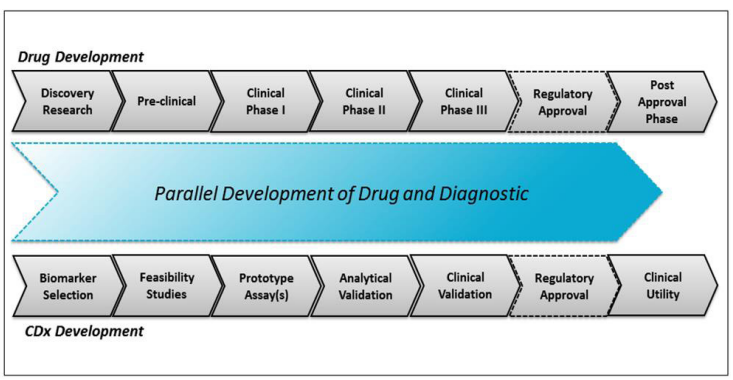

Figure caption: The drug-diagnostic co-development model. The upper part illustrate the drug development process and the lower part the parallel companion diagnostic (CDx) assay development process with an aligned regulatory co-filing at the end of phase III.

Most of the new targeted anti-cancer drugs that have been introduced in recent years, such as BRAF-, ALK-, EGFR- and HER2-inhibitors, are more or less all a product of the drugdiagnostic co-development model. These drugs have shown remarkable high response rates in selected groups of patients within cancer diseases with great unmet medical needs.

This Research Topic on Drug-Diagnostic Co-Development in Oncology aims to provide you with an insight into some of the diverse activities that constitute this new research area.

The front cover is a graphical morphing of two *HER2* amplified breast cancer tissue sections stained with *HER2* CISH pharmDx™ (upper part) and *HER2* FISH pharmDx™ (lower part) kits, respectively. Both assays are FDA approved companion diagnostics. The HER2 CISH pharmDx™ is a companion diagnostic for trastuzumab (Herceptin®, Roche/Genentech). The *HER2* FISH pharmDx™ is a companion diagnostic for trastuzumab (Herceptin®, Roche/Genentech), pertuzumab (Perjeta®, Roche/Genentech), and ado-trastuzumab emtansine (Kadcyla, Roche/Genentech).

Thanks to Dako Denmark A/S for their permission to use the microscopic breast cancer images.

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EDITORIAL
published: 04 August 2014
doi: 10.3389/fonc.2014.00208

Drug-diagnostics co-development in oncology

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Keywords: drug-diagnostic co-development, oncology, companion diagnostics, IHC, FISH, NGS, personalized medicine, precision medicine

The idea of combining drugs and diagnostics in oncology is not new. When the selective estrogen-receptor modulator tamoxifen was developed in the 1970s for the treatment of breast cancer, data on estrogen-receptor status were correlated with the treatment outcome. Based on a phase II study performed in patients with advanced breast cancer, published in 1976, the investigators concluded: "a high degree of correlation between response and positive estrogen-receptor assay suggests the value of the diagnostic test as a means to select patients for tamoxifen treatment" (1). Despite the fact that this conclusion was drawn nearly 40 years ago, the adaptation of the drug-diagnostic co-development model has been relatively slow and it is only within the last decade that it has gained widespread acceptance. The parallel development of the monoclonal antibody trastuzumab (Herceptin®, Roche/Genentech) and the companion diagnostics (CDx) assay for HER2 protein overexpression (HercepTest™, Dako) in the 1990s seems to have served as an inspiration to the pharma and biotech companies (2, 3), and the number of drug-diagnostic co-development projects within oncology has increased rapidly within the last decade.

Genomic sequencing has shown that marked heterogeneity exists in cancer, both between and within patients, which mean that "standard" treatments seldom work for everyone (4). The taxonomy of classifying the cancer diseases, according to their sites of origin and histology, also seems to be far from optimal when it comes to the treatment decision. The philosophy of "one-disease-one-target-one drug" is history and the improvement in cancer pharmacotherapy must come from an increased understanding of the underlying molecular mechanisms in the individual patient. These mechanisms are of a complex nature and we are far from a complete understanding. However, what we do understand is that drugs work at the molecular level, and it is here that we must seek the solution to a more rational drug development process and the subsequent treatment of the patients in the clinic (5). Molecular diagnostic testing has provided us with an increased understanding of the cancer biology, which has recently enabled the development of molecular-based targeted therapies such as vemurafenib (Zelboraf®, Roche/Genentech) for melanoma patients harboring a *BRAF* V600E mutation (6), and crizotinib (Xalkori®, Pfizer) and ceritinib (Zykadia®, Novartis), for non-small cell lung cancer (NSCLC) patients with *EML4–ALK* translocation (7, 8). For the latter two compounds, crizotinib and ceritinib, the development time has been remarkably short, which would never have happened without an in-depth molecular understanding of the disease biology and the mechanism of action of the drugs.

The present research topic of *Frontiers in Oncology* aims to provide an update on the wide-ranging area of drug-diagnostic co-development, biomarker research, and CDx. The research topic covers both basic scientific aspects as well as the clinical and regulatory challenges through a number of Review, Original Research, and Clinical Case Study articles. In the review by Olsen and Jørgensen, an introduction to the subject is given and here both the drug-diagnostic co-development model as well as the clinical and regulatory challenges related to CDx development is discussed (9).

The first CDx to obtain approval by the US Food and Drug Administration (FDA) was the assay for HER2 overexpression (HercepTest™, Dako) based on immunohistochemistry (IHC). IHC is a frequently applied method for protein expression analysis in tumor tissue, and despite the current great focus on gene-based assays, especially next-generation sequencing (NGS), this method is still recognized as an important supplement to analysis of different type of gene aberrations. Likewise, there seems to be cancer-related changes in the proteins that are not directly reflected in the changes in RNA and DNA. Gremel et al. review the currently applied CDx tests based on IHC but points also toward the future with regard to mutation-specific antibodies, *in situ* proximity legation assays, and alternative protein binders such as aptamers (10).

Several articles in this research topic touch upon NGS in relation to CDx, but Pant et al. provide the most comprehensive review (11). In this review, the authors exhaustively discuss the different platforms, sequencing technologies, bioinformatics, data reporting, regulatory aspects as well as the potential use of the technology in relation to drug-diagnostic co-development. There is very little doubt that, in the future, NGS will play a prominent role in the development of molecular-based targeted cancer drugs, however, there is still a number of technical, clinical, and regulatory challenges that needs to be overcome.

The review article by Nicolaides et al. suggests a different approach to drug-diagnostic co-development (12). Here, they discuss the use of co-developing diagnostic-targeting vectors to identify patients whose malignant tissue can specifically take up a targeted anti-cancer drug vector prior to treatment. Using this system, the patients can be predetermined in real-time as to whether or not their tumors can specifically take up a drug-linked diagnostic vector, thus inferring the uptake of a similar vector linked to an anti-cancer agent. According to the authors, this approach offers complementary opportunities to the rapid development of broad tumor-specific agents for use in personalized cancer medicine.

Biomarkers may not only serve as an important tool in relation to development of new molecular-based targeted cancer drugs through the drug-diagnostic co-development model but also for repurposing of existing chemotherapeutic anti-cancer drug. The review article by Stenvang et al. describes a strategy of biomarker-guided repurposing of chemotherapeutic drugs for cancer therapy with a specific focus on the topoisomerase I inhibitors and the use of Top1 as a potential predictive biomarker (13).

The recognition of heterogeneity of cancer diseases has called for a rethinking of the clinical trial designs used to demonstrate safety and efficacy of new targeted anti-cancer drugs. The efficacy of these drugs depends on a specific molecular aberration of the tumor that the drug-diagnostics co-development model tries to encounter. In the review by Simon, different clinical trial designs for the parallel development of drugs and diagnostics are discussed both with respect to the use of a single biomarker as well as a genome-wide discovery of a predictive classifier (14).

The development of crizotinib for treatment NSCLC patients with *ALK* rearrangement is definitively a landmark in relation to drug-diagnostic co-development in oncology. This *ALK* rearrangement was discovered in 2007 and already in 2011 crizotinib obtained US FDA approval together with the FISH assay for detection of this specific rearrangement (Vysis *ALK* Break Apart FISH Probe Kit, Abbott Molecular). In the Review/Opinion by Ou et al., the authors discuss the issue of whether the requirements by the US FDA for the simultaneous co-develop of a CDx will delay the approval of receptor tyrosine kinase (RTK) inhibitors for RTK-rearranged NSCLC (15).

Despite great progress in the treatment of cancer achieved with the use of molecular targeted therapy resistance seems to develop to virtually all of the drugs at some point in time. One way to suppress or delay development of resistance might be through the use of combination therapy. In the review article by Goltsov et al., a rational approach to a systematic development of combination therapies is suggested (16). Based on a joint systems analysis of cellular signaling network response and its sensitivity to drug action and oncogenic mutations, they describe an *in silico* method to analyze the targets of drug combinations.

Resistance is also the issue in the research article by Nielsen et al. where the authors look into the link between miR-21 expression and/or cellular localization and resistance to trastuzumab in HER2 positive patients with breast cancer (17). Tumors from 16 HER2 positive patients who underwent adjuvant treatment with trastuzumab were analyzed. Eight of these patients were considered resistant to the treatment. The result of this small study did not show a link between elevated miR-21 expression and resistance to adjuvant treatment with trastuzumab. However, more studies will be needed in order to prove or eliminate the role of miR-21.

In a clinical case study article by Russell et al., tumor profiling for two patients has been described (18). Both patients had advanced-stage cancer and failed standard treatment. The article describes how tumor profiling was used together with a systematic literature review (Caris Molecular Intelligence™) that was used to identify potential beneficial treatments for the patients resulting in disease remission in both cases.

The use of molecular diagnostics has given us new insight into the cancer disease biology, which has enabled development of new anti-cancer drugs with much more specific and well-defined mechanisms of action. When this knowledge is translated into the drug-diagnostic co-development model, remarkable results can be achieved. Crizotinib is one such example, and a similar or even more remarkable example is the recent development of ceritinib, another ALK inhibitor for NSCLC patients with *ALK* rearrangement. In the spring of 2014, ceritinib obtained an accelerated FDA approval based on efficacy data from only 163 metastatic NSCLC patients enrolled in a phase I single-arm, open-label clinical trial (19). Such a result is only achievable with the use of a CDx that enables pre-selection of the patients who are likely responders to the drug, which as for ceritinib resulted in a response rate above 50% even in a phase 1 trial. Despite the challenges that anti-cancer drug development faces, especially the development of resistance to the molecular targeted drugs, the drug-diagnostic co-development model has shown to be an invaluable tool in oncology, which definitively point to the future.

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Conflict of Interest Statement: Jan Trøst Jørgensen is working as a consultant for Dako and Euro Diagnostica and has given lectures at meetings sponsored by Roche and AstraZeneca.

Received: 22 July 2014; accepted: 22 July 2014; published online: 04 August 2014.
*Citation: Jørgensen JT (2014) Drug-diagnostics co-development in oncology. Front. Oncol. **4**:208. doi: 10.3389/fonc.2014.00208*
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology.

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REVIEW ARTICLE
published: 16 May 2014
doi: 10.3389/fonc.2014.00105

Companion diagnostics for targeted cancer drugs – clinical and regulatory aspects

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Companion diagnostics (CDx) holds the promise of improving the predictability of the oncology drug development process and become an important tool for the oncologist in relation to the choice of treatment for the individual patient. A number of drug–diagnostic co-development programs have already been completed successfully, and in the clinic, the use of several targeted cancer drugs is now guided by a CDx. This central role of the CDx assays has attracted the attention of the regulators, and especially the US Food and Drug Administration has been at the forefront in relation to developing regulatory strategies for CDx and the drug–diagnostic co-development project. For an increasing number of cancer patients the treatment selection will depend on the result generated by a CDx assay, and consequently this type of assay has become critical for the care and safety of the patients. In order to secure that the CDx assays have a high degree of analytical and clinical validity, they must undergo an extensive non-clinical and clinical testing before release for routine patient management. This review will give a brief introduction to some of the scientific and medical challenges related to the CDx development with specific emphasis on the regulatory requirements in different regions of the world.

Keywords: companion diagnostics, *in vitro* diagnostics, drug–diagnostic co-development, regulatory requirements, personalized medicine, precision medicine, oncology

INTRODUCTION

The understanding of the molecular mechanisms of cancer has increased considerably within the last 10–20 years, which has resulted in the development of a number of new targeted drugs. A large proportion of these drugs has been developed using the drug–diagnostic co-development model where the diagnostic test and the drug are developed in parallel (1, 2). The use of this model requires a thorough understanding of the underlying molecular pathology and the drug mechanisms of action, in order to link a certain molecular characteristic to the treatment outcome. The first attempt to use the drug–diagnostic co-development model was made when trastuzumab (Herceptin®, Roche/Genentech) and a immunohistochemistry (IHC) assay were developed for HER2 positive advanced breast cancer (3, 4). Since the approval of trastuzumab and the IHC assay for HER2 overexpression (HercepTest™, Dako) in 1998 by the US Food and Drug Administration (FDA), a number of new targeted cancer drugs guided by a diagnostic assay, a companion diagnostic (CDx) test, has been approved and introduced in the clinic to the benefit of the patients (5). The importance of incorporating a CDx in a drug research project has recently been emphasized by the fact that approximately two-thirds of the breakthrough therapy designations granted by the FDA include a diagnostic assay (6).

The main purpose of developing a CDx assay in most oncology drug research programs is to have a test that can predict whether a patient is likely to benefit from the drug in question. Hence, for many targeted cancer drugs the CDx assays will take up a central role as a kind of "decisive" stratification factor, both during development and subsequently after approval when the drug is used in the clinic. The assay will then become a kind of "gatekeeper" in relation to the treatment decision (2). However, if a CDx assay measures a specific biomarker or combination of biomarkers and it turns out that it is not sufficiently correlated with the clinical state, which could be overexpression of a specific protein or genetic mutations, it will not provide meaningful results. Such an erroneous test result could lead to either a false positive or false negative result, which potentially may cause risk and harm to the patient. For example, a false positive result could lead to treatment with a drug where the biological condition for a positive outcome is missing, and consequently the patient is put at risk due to potential toxic side effects from an ineffective treatment. Similarly, a false negative test result could withhold or delay a potentially beneficial treatment and thereby also bringing the patient at risk (7). In oncology, an early and correct diagnosis and intervention are two elements of key importance in the treatment of cancer patients. In case of a wrong treatment decision, the disease may become disseminated with no or very low chances of cure (2).

The central role of CDx assays in relation to both drug development and the clinical use after approval has caught the attention of the regulatory authorities. Especially the FDA has been at the forefront in relation to developing regulatory strategies for drug–diagnostic co-development and personalized medicine. As described above, it is important to avoid false positive and false negative test results and the analytical and clinical validity of any CDx assay must be sufficiently documented before it can be

approved for routine use in the clinic (1, 7). In this article some of the scientific and medical challenges related to the CDx development are discussed with specific emphasis on the regulatory requirements.

COMPANION DIAGNOSTICS – TERMINOLOGY AND DEFINITIONS

With regards to the terminology and definitions of a diagnostic assay that is developed in parallel to a targeted drug and used to guide the treatment decision, there seems to be lack of consensus. Different names are used in the literature, such as pharmacodiagnostics, theranostics, pharmacogenomic biomarkers, and companion diagnostics. Within the last few years, the name companion diagnostics has been used more and more frequently and this is also the term that has been adapted by the FDA and now also the European Union (EU), however, theranostics is still used quite frequently especially in the academic literature (2). In 2011, the FDA issued a draft guidance on *In vitro Companion Diagnostics Devices* where a CDx was defined (8). According to this definition a CDx assay is an *in vitro* diagnostic device that provides information that is essential for the safe and effective use of a corresponding therapeutic product. Further, the FDA specifies three areas where a CDx assay is essential: (1) to identify patients who are most likely to benefit from a particular therapeutic product; (2) to identify patients likely to be at increased risk of serious adverse reactions as a result of treatment with a particular therapeutic product; and (3) to monitor response to treatment for the purpose of adjusting treatment (e.g., schedule, dose, discontinuation) to achieve improved safety or effectiveness. So according to the FDA, a CDx assay can be used both to predict outcome (efficacy and safety) and to monitor the response.

The definition that has been proposed by the EU is somewhat narrower and is more or less limited to item 1 in the FDA definition. According to the proposed regulation on *in vitro* diagnostic medical devices from 2012, a CDx is a device specifically intended to select patients with a previously diagnosed condition or predisposition as eligible for a targeted therapy (9). With no doubt the predictive or selective characteristics of a CDx assay has so far attracted the most attention. The use of a CDx assay facilitates the design of clinical trials with a smaller number of subjects, which has a positive effect on the resources and time spent on clinical development (2). A definition that focuses on the predictive or selective characteristics of the CDx assay and makes a link to "personalized medicine" is: "A pre-treatment test performed in order to determine whether or not a patient is likely to respond to a given therapy. This type of test is classified as a predictive or selective test and is a prerequisite for implementation of personalized and stratified medicine" (10).

DRUG–DIAGNOSTIC CO-DEVELOPMENT

In the drug–diagnostic co-development model there is interdependency of drug and diagnostics. The CDx assay is developed in parallel to the drug, as illustrated in **Figure 1**. The success of such a co-development project depends very much on the strength of the biomarker hypothesis, which is often deduced during the early research and preclinical phases of the drug development. As previously mentioned, it requires a thorough molecular understanding of both the pathology and drug mechanisms of action to come up with a solid hypothesis. It might not only be one hypothesis which is tested through prototype assays but several hypotheses. These prototype assays are subsequently used during the clinical phases I and II in order to give an idea of the predictive potential.

If one or more of these hypotheses appears promising the assay will then undergo analytical validation. However, before the analytical validation of the CDx assay can be finalized, the cut-off value must be established, which is usually done based on outcome data from phase I/II clinical trials. During the analytical validation, it must be demonstrated that the assay accurately and reliably measures the biomarker that has been selected earlier on in the development process. In relation to this validation, a number of both internal and external studies must be performed. For the external analytical validation a multi-site study is performed to document reproducibility using the final version of the CDx assay across several laboratories. Before using the CDx assay for patient selection and treatment stratification in a clinical phase III trial,

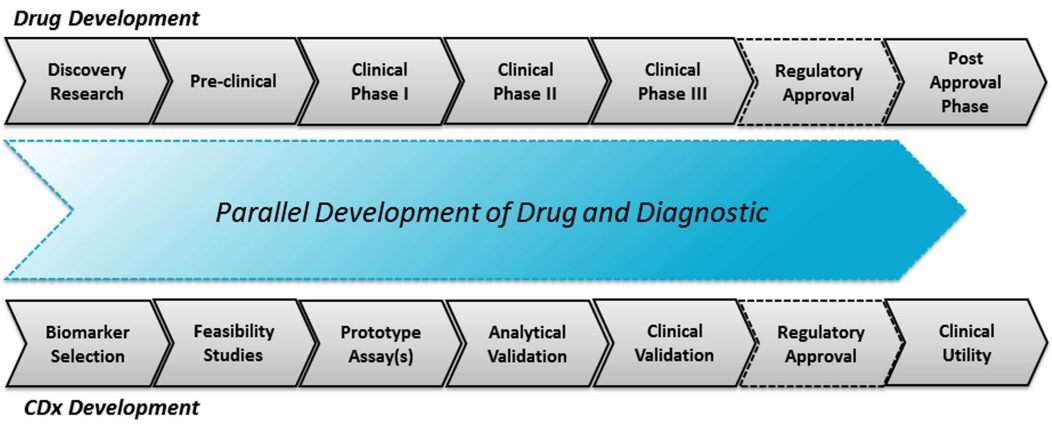

FIGURE 1 | The drug–diagnostic co-development model. The upper parts illustrate the drug development process and the lower parts the parallel CDx development process with an aligned regulatory co-approval at the end of phase III.

Table 1 | Overview of the main clinical trial designs that have been proposed for the parallel development of drugs and diagnostics. The last column in the table lists the diagnostic metrics that can be calculated based on the given clinical trial design. CDx+, test positive patients; CDx−, test negative patients; PPV, positive predictive value; NPV, negative predictive value.

Clinical trial design	Description	Diagnostic metrics
All-comers*	All patients meeting the study eligibility criteria are enrolled in the trial independent of the CDx test results	Sensitivity, specificity, PPV, and NPV
Enrichment	Only patients who are CDx+ and meet the study eligibility criteria are enrolled in the trial	PPV
Stratified	Both CDx+ and CDx− patients meeting the study eligibility criteria are enrolled in the trial and subsequently randomized	Sensitivity, specificity, PPV, and NPV

**Low prevalence of CDx+ patients requires a large sample size.*

it is strongly recommended that the assay is analytically validated (1, 7). Due to challenges with respect to the alignment and timing of the development of the drug and the CDx assay, it is sometimes tempting to start the clinical trial with a prototype assay and then replace it with the validated version later on during the trial. However, such a strategy is not recommendable as it makes it difficult to interpret the clinical trial results due to the fact that the patients have been selected using two different versions of the assay (7). If different versions of an assay have been used during clinical validation a subsequent bridging study will be needed, which is both resource demanding and time consuming. A "golden rule" with regards to the final clinical validation of a CDx is to use only one version of the assay, which is the analytically validated version, and only one testing laboratory in order to reduce possible site to site variation.

In the drug–diagnostic co-development model, phase III is not only used to demonstrate safety and efficacy of the drug, but also to clinically validate the CDx assay. Here, it must be demonstrated that the CDx assay has an ability to predict the treatment outcome in the individual patients (7). A CDx assay will only be useful if it provides information that can discriminate between patients who are likely responders and non-responders, and in this respect the clinical diagnostic accuracy of the assay is important, thus data on the clinical sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the CDx assay are important diagnostic metrics to consider. Several trial designs for clinical drug–diagnostic co-development have been proposed, however, not all of them make it possible to calculate the described diagnostic metrics. **Table 1** provides a brief overview of the main clinical trial designs that have been proposed for the parallel development of drug and diagnostic, however, in this article only the enrichment design will be discussed, as it is the design that so far has been used most frequently in relation to drug–diagnostic co-development. Furthermore, a relatively large number of review articles and draft guidance document have been published within the last few years describing these trial designs in more details (1, 2, 11–14).

The enrichment trial design is often used if there is clear evidence of a strong relationship between a positive CDx status and the treatment outcome with the targeted drug (e.g., from previous phase I/II studies) (1, 2). With this design, all the patients are tested by means of a CDx assay, but only the CDx positive patients are enrolled in the study and subsequently randomized to either the new targeted drug or to the standard treatment, as shown in **Figure 2**. The advantage of this design is that it generally requires a smaller number of patients to be randomized compared with the all-comers design, due to the fact that only patients who have a CDx positive status are enrolled in the trial, thus making the study population more homogeneous. However, this design allows only the PPV to be calculated and not sensitivity, specificity, and NPV, which is a limitation of this a trial design (1, 2). The enrichment design was also the one used when trastuzumab went through final phase III testing in women with advanced breast cancer in the 1990s (3). Further, looking at the drug–diagnostic combinations that have obtained FDA approval, the enrichment design is the most frequently used to demonstrate safety and efficacy of the drug and to clinically validate the corresponding CDx assay. Recent examples of targeted cancer drugs that have used this trial designs are vemurafenib (Zelboraf™, Roche/Genentech), crizotinib (Xalkori®, Pfizer), pertuzumab (Perjeta®, Roche/Genentech), ado-trastuzumab emtansine (Kadcyla®, Roche/Genentech), dabrafenib (Tafinlar®, GSK), and trametinib (Mekinist™, GSK). A list of the CDx assays and their corresponding therapeutic product that have been approved by the FDA can be found at the webpage of Center for Devices and Radiological Health (CDRH) (5).

How effective is the use of a CDx in the drug development process? This question was partly answered in an analysis made to estimate the risk of clinical trial failure during non-small cell lung cancer (NSCLC) drug development in the period between 1998 and 2012 (15). The data material was retrieved from different available public sources and 676 clinical trials with 199 unique drug compounds meeting the inclusion criteria of the analysis. The data showed that the success of clinical phase III was the biggest obstacle for drug approval with an overall success rate of only 28%. A small improvement in the success rate was found for the receptor targeted therapies tested in phase III. However, the absolutely highest success rate was observed when the drug was biomarker-guided showing a success rate of 62%, as seen in **Figure 3**. So, the conclusion from this analysis indicates that the use of a CDx assay during phase III drug development improves the success rate considerably. The data from this analysis also seem to confirm the effectiveness of the enrichment design described earlier in this paragraph.

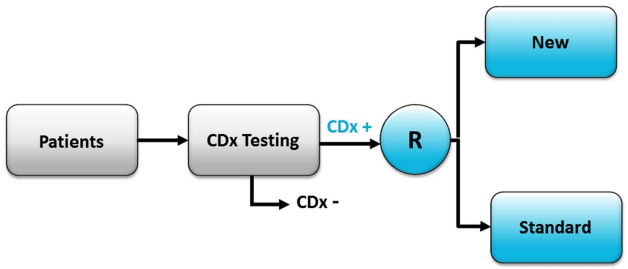

FIGURE 2 | The enrichment clinical trial design. With this design only patients who have a positive CDx assay result are enrolled in the trial and randomized (R) to either the new targeted treatment (New) or standard treatment (Standard). CDx+, indicates test positive patients; CDx−, indicates test negative patients.

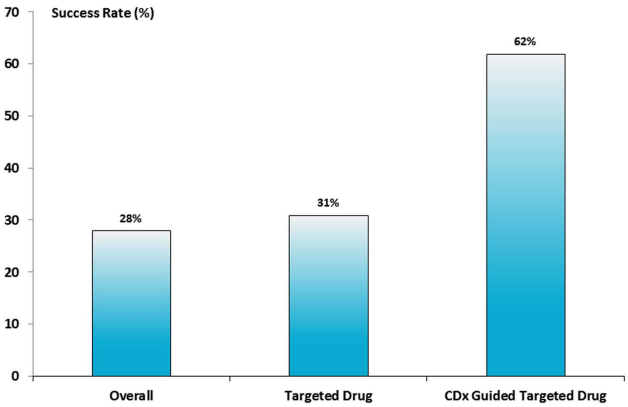

FIGURE 3 | Success rate for NSCLC drugs in phase III clinical trials. Based on the analysis of 676 clinical trials a success rate of 28% was found for all types of drugs, however, if the drug was either a receptor target drug or guided by a CDx assay the success rate increased to 31 and 62%, respectively (15).

COMPANION DIAGNOSTICS AND REGULATORY REQUIREMENTS

Recent developments in the field of personalized medicine and drug–diagnostic co-development have been most challenging, not only for the regulatory professionals but also for regulatory authorities. While drug companies and CDx manufacturers found new grounds in collaboration to jointly bring their products to patients, some regulatory authorities have been too slow to adapt to the changing regulatory landscape caused by the CDx development. A few highlights of the regulatory process for CDx in the major markets are presented and discussed below.

FDA SETTING THE STANDARD FOR REGULATORY PATHWAY OF COMPANION DIAGNOSTICS

In April 2005, the FDA published the Drug–Diagnostic Co-Development Concept Paper. This document labeled by the FDA "Draft Preliminary Concept Paper – Not for Implementation" has become a landmark for the formalization of the drug–diagnostic co-development strategy (1). Even though the pharma and diagnostic companies have seldom found the co-development model for parallel development of drug and diagnostic feasible, it provided grounds for alternative development strategies and for obtaining FDA feedback prior to initiating non-clinical or clinical testing, or prior to intended submission of a marketing application. Since 2005, the FDA has taken the lead and set the standard for the CDx regulatory pathway. This standard also provided inspiration to other authorities and regulatory professionals worldwide. The FDA further strengthened its leading position in defining the regulatory landscape for CDx by creating a personalized medicine group within the Office of *In vitro* Diagnostics and Radiological Health (OIR), formerly, Office of *In vitro* Diagnostics (OIVD) in 2009. This group has contributed to a considerable number of guidance documents related to CDx. Furthermore, FDA is providing transparency of the approval process by including web availability of Safety and Effectiveness Summary documents for the approved CDx.

While waiting for an update of the 2005 concept paper, a draft of the *In vitro* Companion Diagnostic Devices guidance was published in July 2011 (8). This guidance document is not a replacement of the 2005 concept paper, but rather an operational guide for *In vitro* Diagnostics (IVD) of the pharma and biotech industries indicating possible regulatory pathways as well as labeling and regulatory requirements for CDx devices and therapeutic products (5).

Attention should be paid to an important section of the guidance covering the investigational use of CDx. Before the "companion diagnostics era," many of the investigational IVD devices were either exempted from Investigational Device Exemption (IDE) regulations or classified as non-significant risk devices subject to abbreviated IDE requirements. In the case of clinical trials, where companion diagnostics are used to make a medical decision – such as treatment assignment, an IVD is considered a serious risk device requiring IDE approval by the FDA. Typically, a pharma or a biotech company is the sponsor of a drug–diagnostic clinical trial conducted under the Investigational New Drug (IND) regulations. However, it is important that an IDE for the diagnostic is either included in the IND or submitted and approved separately. According to the guidance document, FDA accepts that the IDE information is included in the IND. However, as the IDE format is not compatible with an IND, in some cases, the FDA has expressed that a separate IDE is preferred (16). Hopefully, this will be further clarified in the final version of the guidance, which is expected by October 2014 (17). The content of an IDE is well-defined in the regulations and further specified on the FDA website and in several guidance documents (18). In the case of a combined drug–diagnostic clinical trial, the IDE must, in addition to information on the CDx assay, also include information provided by the drug sponsor, such as the clinical trial protocol, investigational sites, Investigational Review Board (IRB) information, and informed consent material for patients. Thus, in relation to collaboration between a drug company and a diagnostic company, it is important that roles, responsibilities, and timelines are clearly defined between the parties.

In the 2011 guidance document, the FDA declares that "the FDA review of the test/therapeutic product pair will be carried out collaboratively among relevant FDA offices." Truly, FDA offices

responsible for each of the products are not only collaborating in the review process but are also announcing approvals of both the drug and CDx concurrently.

Another important guidance, not only for CDx, is the Medical Devices: the Pre-Submission Program and Meetings with FDA Staff published in draft in July 2012. Final version of the guidance was published in February 2014 (19). In this document, the Pre-IDE program was renamed to a Pre-Submission (Pre-Sub) program (19). Since 1995, the Pre-IDE, now Pre-Sub, program, has allowed industry to obtain FDA feedback prior to any kind of device submission and thus providing opportunities for the industry to discuss a drug–diagnostic co-development strategy at any development or testing stage. Even though there is no user fee for a Pre-Sub, the process has become more formalized since authorization of the Medical Device User Fee and Modernization Act (MDUFMA) in 2012 (20). In the new guidance, the FDA provides recommendations to the contents of the Pre-Sub and also clarifies the administrative procedures of the program. The Pre-Sub is a formal, written request for feedback from the FDA regarding analytical or clinical study protocols or a proposed regulatory pathway. A Pre-Sub may also be an appropriate way to acquaint the FDA with a novel technology or design. A Pre-Sub interaction with the FDA is a particularly useful way to discuss testing strategies which are not the ideal co-development scenarios, and where an analytically validated assay is not available at an early stage of the clinical drug development. The benefits of Pre-Subs may include time and cost reduction of research or clinical studies, better understanding of FDA expectations and trends, especially in the area where no guidance documents are available, and, most importantly, may result in a better and more complete marketing application and greater chance of a successful approval. In order to improve the understanding of IVD related issues, it is recommended that the drug sponsor participates in the Pre-Sub process initiated by the diagnostic company. If relevant, CDRH will request Center for Drug Evaluation and Research (CDER) attendance in the process. According to the FDA statistics, the inter-center consultations have increased from 39 in 2010 to 106 in 2012 (16), which is a likely consequence of the increased number of drug–diagnostic co-development projects mainly within oncology.

The controls required by the FDA prior to marketing of a device in the US depend on the classification of the device. Medical devices, including IVDs, are risk-classified as class I, II, or III. The majority of companion diagnostic IVDs are high risk class III devices. This review will not go into details of the regulatory requirements for each product class, but only give a very brief summary. For class I devices, general controls, like establishment registration and device listing, apply; for class II devices, general controls and a premarket clearance [510(k)] is needed; and class III devices require the most stringent approval for medical devices by the FDA, a Premarket Approval Application (PMA) (21).

Briefly, a PMA application may be either traditional or modular. There is no difference in the contents of a traditional or a modular PMA but there is a difference in the way the PMA is submitted for FDA review. In a traditional PMA, all information required by the regulations is submitted at the same time, while for a modular PMA the information is submitted in modules. Thus, analytical performance (non-clinical studies) and manufacturing information may be submitted and reviewed by the FDA while a clinical trial is ongoing. When the clinical trial is completed, the data will then be submitted to the FDA. At this point of time, the other modules have been through FDA review. This approach may allow for a shorter approval process and a better alignment with the drug approval.

A modification in an intended use for a PMA-approved CDx, such as adding a new indication or a new targeted drug, is a complex process which, depending on the type of modification, may require massive analytical and/or clinical data. The FDA is very responsive to Pre-Subs for device modifications and provides feedback to proposed regulatory pathways and studies supporting regulatory submission for the change in the intended use of the specific CDx.

EUROPE TIGHTENS UP THE IVD LEGISLATION

The IVD Directive 98/79/EC regulates *in vitro* diagnostic medical devices in the EU, EU candidate countries, and associated countries (22). The current EU regulatory framework for IVD devices demonstrates how unnoticed CDx IVD devices were at the end of the nineties when the IVD Directive was proposed and subsequently entered into force in 2003. There is no specific mention of CDx in the definition of an IVD, and the classification system of the directive does not consider CDx at all. Also, the IVD Directive list-based classification system has shown its limitations, as only a limited number of IVD devices are considered medium or high risk devices (so-called Annex II devices). All remaining IVDs, including CDx assays, are classified as low risk devices. Adding a new device to the Annex II list has proved to be a cumbersome process. It has taken 4 years to add a variant Creutzfeldt–Jakob disease assay to List A of Annex II, which the United Kingdom requested in 2007, and the decision from the EU Commission came only in 2011 (23).

Briefly, IVD devices placed in the EU market require a CE-mark to indicate conformity with the IVD Directive. For the high risk products listed in Annex II, the involvement of a Notified Body (NB) is required to assess conformity to the IVD Directive before placing the device in the European market. An NB is an organization accredited by a member state to assess the manufacturer's conformity to the essential requirements of the directive.

Currently, any CDx assay entering the EU market is classified as low risk device based on a conformity assessment and CE-marking by the manufacturer, the so-called self-certification procedure. This results in incomprehensible differences in the regulatory pathway to the market between the USA (PMA approval) and the EU (self-certification).

However, there are major changes under way in EU IVD medical device legislation, which will impact CDx assays entering the market. The IVD Directive will be replaced by a Regulation on IVD (9). A regulation is the most powerful, single regulatory framework, which is applicable in a uniform manner at the same time for all EU member states, which leaves no room for divergent transpositions.

A draft of the new IVD Regulation (IVDR) has already been proposed, and obviously, we will be facing a very different regulatory landscape in the EU in the years to come (9). In the

classification system proposed in the IVDR, IVDs will be assigned to four classification groups A, B, C, and D, depending on device risk, with class A being the lowest risk class. The four-class system resembles what we already know from the Canadian and Australian regulations, and is similar, but not equal, to what has been proposed by a Global Harmonization Task Force (24). CDx assays will be Class C devices and will require a complex regulatory pathway including a requirement for a Design Examination Certification by an NB. The review by the NB may possibly also be linked to a consultation with the European Medicines Agency (EMA) or, alternatively, compliance to a Common Technical Specifications (CTS) will be required. The CTS for new devices will be drafted as part of the review process. No matter which of the proposed pathways (EMA consultation or CTS) becomes final, the time to the market for a CDx assay will be extended essentially. It is assumed that the proposed IVDR will pass through the Council and Parliament in 2014, and the Regulation will then enter into force in 2017, after a 3-year implementation period.

JAPAN EXPECTED TO MIRROR FDA REVIEW AND APPROVAL PROCESS

In Japan CDx assays are classified as high risk devices (class III), however, the regulatory approval process has until now been disconnected from the approval of the related therapeutic products. In October 2011, the Japan Association of Clinical Reagent Industries (JACRI) addressed the Ministry of Health, Labour and Welfare (MHLW) and the Pharmaceuticals and Medical Devices Agency (PMDA) with a proposal for a regulatory pathway for companion diagnostics (25). At the end of December 2013, the final guidance for CDx and related drugs was announced in the PMDA Notification. The guidance includes a CDx device definition, guidance for application for CDx and therapeutic products, clinical studies of therapeutic products as well as a review system by PMDA. In the original proposal presented to the MHLW/PMDA, JACRI has taken into consideration the FDA draft guidance on *In vitro* Companion Diagnostic Devices that was issued in July 2011 (8). Thus, the final PMDA guidance stresses that application for both a CDx and its corresponding therapeutic product should be submitted and reviewed at the same time under PMDA. Furthermore, it is recommended that drug and diagnostic sponsors seek early consultation with the authorities on the regulatory pathway for CDx, similar to the FDA Pre-Sub program. It is expected that publication of the guidance will improve the review process for companion diagnostics IVDs in Japan and make it more transparent. There is no English version of the guidance available at the moment on the PMDA website (26).

CHINA REGULATORY PATHWAY IS A CHALLENGE

Requirements for registration of IVD devices in China exceed requirements in any of the other countries and regions described above. Here, CDx assays are as in the US and Japan, classified as high risk devices (class III products). So far, there have been no guidance documents issued for CDx assays, but the registration process follows the requirements for class III products and requires extensive documentation and supporting testing data to be submitted to the China Food and Drug Administration. Specific for a class III IVD device in China, there is a requirement for local testing of at least 1000 patient specimens divided among three geographically distinct hospitals. This testing must be performed using three consecutive lots of the device and further detailed lot records, including specific requirements for stability testing and analytical performance testing must be provided. In addition, a number of legal documents are required to be submitted such as legal qualification of the manufacturer and authorization letters for authorized representatives.

CONCLUSION AND FUTURE CONSIDERATIONS

Companion diagnostics holds the promise of improving the predictability of the oncology drug development process and become an important tool for the oncologist in relation to the choice of treatment for the individual patient. A number of drug–diagnostic co-development projects have already been completed successfully, and in the clinic, the use of several targeted cancer drugs are now guided by a CDx. For these drugs the management of the patient partly depends on the result generated by the CDx assay, and consequently this type of assay has become critical for the patient care. In order to avoid "false positive" and "false negative" test results, it must be documented for any CDx assay that it has a high degree of analytical and clinical validity (7). To some extent this is comparable to the safety and efficacy documentation that needs to be generated in order to achieve a marketing authorization for a new drug (27).

The central role of the CDx assays in relation to the current and future pharmacotherapy has attracted the attention of the regulators, especially the US FDA. In the US, CDx assays are in most cases classified as class III, high risk devices, for which the most stringent requirement for safety and effectiveness documentation apply, including submission of a PMA. Knowing the critical role of a CDx assay in relation to patient management this seems only reasonable that a number of other countries including Australia, Canada, China, and Japan have followed suit with regards to stringent requirements. However, for the EU, it has taken some time to realize the critical importance of CDx assays in relation to patient care and safety, and only recently the discussions about a more up to date regulation for IVD medical devices including CDx assay has started. Despite the coming new legislation in the EU not seeming to have the same formalized co-development and co-approval process as in the US, it will most likely increase the patient safety.

Many of the biological characteristics important for a specific drug to be effective, such as mutations, gene rearrangements, gene amplifications, and protein overexpression are typically not present in one cancer type alone, but are often found across several cancer diseases. *HER2* amplification and protein overexpression are such examples, where these characteristics are found in breast and gastric cancer as well as others cancers. Further, it has also been shown that an HER2 targeted drug like trastuzumab is effective in both breast and gastric cancer (3, 28). This and other examples have shown what matters most in relation to determining the response to a specific drug is the molecular pathways driving the growth of the cancer and not from where in the body the tumor originates. Based on this knowledge, we will probably see drug–diagnostic combination being developed for several cancer diseases simultaneously in the future, which will be both scientifically and medically challenging. How the drug regulatory system,

such as the FDA, will handle this challenge will also be interesting to see, as both drugs and CDxs have been approved for one cancer disease at a time up to now.

Most of the CDx guided targeted cancer drugs that have been introduced within the last few years have shown significantly high response rates and prolonged progression free survival in specific selected groups of patients. Previously, for many of the treated patients no treatment has been available for their specific disease, and CDx guided drugs definitively represent a real progress within oncology. However, for all these drugs, resistance will develop at some point in time resulting in disease progression. For this reason it is unlikely that "monotherapy" with a targeted cancer drug based on identification of a single biomarker will achieve long-lasting remission, and we will probably need to move away from the "one biomarker one drug" model toward a more multimodal approach (29). This new model will need to integrate multiple biomarkers and multiple targeted cancer drugs and should be based on a simultaneous use of several drugs in order to block more signal pathways, thus to prevent resistance to develop. When it comes to CDx assays, this will make a call on specifically designed multiplex assays most likely based on technologies such as gene expression arrays or next generation sequencing (NGS) (30). Despite the very recent decision by the FDA to grant marketing authorization for the Illumina instrument platform for screening and diagnosis of cystic fibrosis, there still seems to be a number of challenges that must be overcome before we see NGS as CDx for targeted cancer drugs (31, 32). However, the advantages of this type of technology are that they will enable researchers and healthcare professionals to get a broader look at the cancer patients' genetic makeup and probably help them designing more effective treatment modalities. Several CDx possibilities seem to be available to improve the treatment of the cancer patients, however, the development of assays will face a challenging time both with respect to medical/scientific as well as regulatory aspects.

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Conflict of Interest Statement: Jan Trøst Jørgensen is working as a consultant for Dako and has given lectures at meetings sponsored by Roche and AstraZeneca. Dana Olsen is an employee of Dako.

Received: 19 February 2014; accepted: 28 April 2014; published online: 16 May 2014.
*Citation: Olsen D and Jørgensen JT (2014) Companion diagnostics for targeted cancer drugs – clinical and regulatory aspects. Front. Oncol. **4**:105. doi: 10.3389/fonc.2014.00105*
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology.

frontiers in ONCOLOGY

REVIEW ARTICLE
published: 31 October 2013
doi: 10.3389/fonc.2013.00271

In situ protein detection for companion diagnostics

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The emergence of targeted therapies for cancer has created a need for the development of companion diagnostic tests. Assays developed in recent years are aimed at determining both the effectiveness and safety of specific drugs for a defined group of patients, thus, enabling the more efficient design of clinical trials and also supporting physicians when making treatment-related decisions. Immunohistochemistry (IHC) is a widely accepted method for protein expression analyses in human tissues. Immunohistochemical assays, used to localize and quantitate relative protein expression levels within a morphological context, are frequently used as companion diagnostics during clinical trials and also following drug approval. Herein, we describe established immunochemistry-based methods and their application in routine diagnostics. We also explore the possibility of using IHC to detect specific protein mutations in addition to DNA-based tests. Finally, we review alternative protein binders and proximity ligation assays and discuss their potential to facilitate the development of novel, targeted therapies against cancer.

Keywords: companion diagnostics, immunohistochemistry, Her2, alternative binders, proximity ligation assays

INTRODUCTION

Throughout recent decades, our understanding of the molecular basis of cancer development has dramatically improved. This is reflected in the growing number of targeted cancer therapies and significantly affects today's standard of care in oncology. Nevertheless, a prerequisite for an effective, targeted cancer treatment concerns the selection of patient, which creates a growing demand for reliable companion diagnostic devices. The rationale behind such developments is to ensure that treatment is not withheld from patients whom it may benefit while at the same time protecting them from overtreatment, the risk of unnecessary side effects and, most importantly, a delay in receiving treatment with a more suitable agent.

Companion diagnostics also play an important role during the pre-clinical stages of drug testing. A potent effect observed in a small patient population may be missed by the absence of a reliable companion diagnostic test. Conversely, a novel subset of patients may be found to benefit from treatment or no difference in efficiency may be detected, regardless of biomarker positivity. These issues pose a challenge to the parallel development of drug and companion diagnostic tests and consequently, the latter should be fully validated before the initiation of clinical trials and the trial design adjusted accordingly (1). Despite much advances, the corresponding regulatory framework is still incomplete and while the US Food and Drug Administration (FDA) dictates a stringent premarket approval procedure for all companion diagnostic devices, similar legislation is still under review in Europe (1). Currently, only 19 companion diagnostic devices have been approved by the FDA, 10 of which are intended for the detection of the human epidermal growth factor receptor 2 (ERBB2, also referred to as HER2)[1].

[1]http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm

Most companion diagnostic tests used in a clinical setting are based on immunohistochemistry (IHC), real-time reverse transcription PCR (qRT-PCR), or *in situ* hybridization (ISH). With regard to ISH, assay systems based on either fluorescent (FISH) or colorimetric (CISH) signal detection have been established and each testing modality is associated with a number of advantages and disadvantages (**Table 1**).

While the scope of companion diagnostics is broad, with this review we will focus on techniques designed to detect proteins in formalin-fixed, paraffin-embedded (FFPE) tissue. We will discuss currently applied companion diagnostic tests which use IHC and also novel developments regarding mutation-specific antibodies, *in situ* proximity ligation assays (PLA), and alternative protein binders.

APPLICATION OF IMMUNOHISTOCHEMISTRY IN CLINICALLY USED COMPANION DIAGNOSTICS

ESTROGEN RECEPTOR

The introduction of tamoxifen, a selective estrogen receptor (ER) modulator, over 30 years ago has revolutionized the clinical management of breast cancer. However, since significant treatment benefits were only observed in ER-positive patients (2, 3), companion diagnostic testing became imperative. Originally, various ligand binding assays (LBAs) were used to quantify the expression of ER, however, they required homogenization of fresh frozen tumor material and were thus laborious in their execution. With the development of monoclonal antibodies targeting the ER and antigen retrieval methods for the use of FFPE tissue, LBAs were soon replaced and IHC became the standard diagnostic tool. Several grading systems were subsequently introduced to describe IHC-based ER expression levels, including (1) Allred score (range: 0–8) (4), (2) Quick score (range: 0–7) (5) (both of which are based on the sum of fraction and intensity units of the stained cells), (3) *J*-score (range: 0–3, based on the fraction of stained cells) (6)

Table 1 | Advantages and disadvantages of currently used companion diagnostic techniques.

Technique	Advantage	Disadvantage
IHC	Routinely performed; low technological requirements; time and cost effective; preservation of histological information; suitable for small tumor samples	Semi-quantitative; subjective interpretation of results; variability dependent on fixation procedure, staining protocol, and antibody selection
qRT-PCR	Quantitative; large dynamic range	No histological information retained; contamination of test results by stromal/normal tissue possible; increased technological requirements; increased time and cost requirements; variability dependent on tissue quality, RNA extraction/processing procedures and primer/probe selection
ISH	Quantitative for genetic alterations; higher reproducibility	Increased technological requirements (especially for FISH); increased time and cost requirements; added expertise in result interpretation necessary

IHC, immunohistochemistry; qRT-PCR, quantitative real-time PCR; ISH, in situ hybridization; FISH, fluorescent in situ hybridization; CISH, colorimetric in situ hybridization.

and (4) H-score (range: 0–300, based on the product of fraction and the intensity unit of stained cells) (7). Differences between positive/negative definitions together with variations regarding antibody clones, tissue fixation, antigen retrieval, and detection protocols, all contributed to a significant rate of variability in ER detection (8–10). For instance, a study headed by the UK National External Quality Assessment Scheme for Immunocytochemistry (NEQAS-ICC) reported false negativity rates ranging from 30 to 60% following the testing of a standardized sample with low ER expression by 200 laboratories throughout 26 countries (8).

Due to the historic nature of ER testing, there are currently no FDA-approved companion diagnostic devices available. To minimize inter-laboratory variation, the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) have recently published a document outlining their recommendations for the immunohistochemical testing of ER in breast cancer (11). The optimal testing conditions and tissue handling requirements were defined together with guidelines for both the internal and external quality assurance procedure. The same guidelines were also applicable to the detection of the progesterone receptor (PR) via IHC. PR is located downstream of ER and a positive PR test result may be indicative of an intact estrogen signaling cascade (12). This line of thinking was corroborated by the finding that patients with PR positive tumors had a better prognosis than patients with ER-positive/PR negative breast cancers (13). Alternative methods for the detection of ER and PR are continuously under investigation and Oncotype DX is one such example. This is a qRT-PCR-based assay system designed to estimate the probability of distant tumor recurrence in tamoxifen-treated, node-negative breast cancers (14) and measures the expression PR and ER, together with 19 other genes, in mRNA extracted from FFPE tissue. Proof-of-concept studies on the applicability of Oncotype DX as a companion diagnostic tool for tamoxifen treatment reported varying conclusions. In a study supported by Genomic Health Inc., Badve and colleagues reasoned that ER/PR determination using Oncotype DX performed comparably well to IHC-based detection systems (15). This was in contrast to an independent evaluation by Kraus et al. who concluded that IHC was superior to the Oncotype DX qRT-PCR-based test, not only due to higher sensitivity but also the lower cost, the ease of application and the preservation of morphological information (16).

HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR 2

Overexpression of the HER2 protein and/or amplification of the HER2-encoding gene have been associated with an unfavorable prognosis in several types of cancer, including breast, gastric, and pancreatic cancer (17–19). Trastuzumab (Herceptin), an antibody-based inhibitor, was the first HER2-targeted drug to be approved for the treatment of breast cancer and has since been shown to significantly improve survival in a metastatic and adjuvant setting (20, 21). Numerous studies support the close relationship between HER2 positivity and trastuzumab responsiveness (20, 22) and bearing in mind that a HER2-amplification rate of approximately 25% occurs in breast cancer, initial clinical trials may not have yielded significant data had no pre-selection of patients according to HER2 status taken place (23). Since gene-amplification is the primary cause of HER2 overexpression (24), both FISH- (or CISH-) and IHC-based companion diagnostic devices have been approved by the FDA. Nevertheless, the initial evaluation of HER2 status is usually performed using an IHC-based method and only ambiguous (or equivocal) cases are subjected to FISH reflex testing.

Substantial inter-laboratory variations in test results are an inherent problem when considering IHC-based tests. Similar to the guidelines established for ER, ASCO/CAP has produced recommendations for HER2 testing in breast cancer (25, 26). In contrast to the instructions for the commonly used FDA-approved HercepTest (Dako), which states that a finding of more than 10% of cells with strong, uniform membrane staining qualifies as a positive result, the ASCO/CAP guidelines require complete intense membrane staining in >30% of cells in order to qualify as a positive test result following IHC testing. In addition, unlike the FDA-approved cut-off ratio of 2.0 for HER2/Chromosome 17 centromere (CEP17) testing via FISH (or four HER2 copies in assays without internal CEP17 probes), ASCO/CAP considered the range of ratios between 1.8 and 2.2 (or four to six HER2 copies) as equivocal and stated that only cases with HER2/CEP17 ratios >2.2

(or more than six copies of HER2) could be deemed to be positive based on FISH analysis. The aim of the new ASCO/CAP guidelines was to reduce the number of inconclusive cases and although some groups were positive to these new definitions (27, 28), others saw no added benefit (29). While high concordance between IHC- and FISH-based HER2 testing was demonstrated by several studies, thus justifying the use of routine IHC as an initial test (30), critics are keen to highlight the technical superiority of FISH over IHC and consequently advocate FISH as the gold standard for HER2 testing (31). Hence, it is evident that more conclusive studies on the clinical significance of both testing modality are required.

EPIDERMAL GROWTH FACTOR RECEPTOR

The epidermal growth factor receptor (EGFR) is a prominent therapeutic target in both colorectal and non-small cell lung cancer (NSCLC). In colorectal cancer, it is primarily targeted by the monoclonal antibody-based drugs cetuximab and panitumumab. These drugs target the extracellular domain of the EGFR and block down-stream signaling. Clinical trials for both agents initially required the pre-selection of patients based on positive expression of the EGFR protein, as determined by IHC, however, it soon became evident that IHC-based protein expression levels did not correlate with therapy outcome (32, 33) and that even patients with EGFR-negative tumors may benefit from EGFR-targeted therapy (34, 35). The causes for this discrepancy may be of a technical nature and connected to the variability/sensitivity of immunohistochemical techniques, or they may be a direct consequence of biological determinants. For instance, metastatic tumors may have lost the expression of EGFR, rendering them unresponsive to therapy (36). In addition, Chung and co-workers reasoned that antibodies used within IHC-based detection systems were unable to discriminate between high- and low-affinity EGFRs. Consequently, the relative distribution of such high- and low-affinity EGFRs within colorectal cancer tissue may be crucial in determining the response to therapy (34). Furthermore, it has been noted that therapeutic antibodies that target EGFR may induce antibody-dependent, cell-mediated cytotoxicity, resulting in an indirect beneficial effect owing to the recruitment of cytotoxic immune cells such as monocytes and natural killer cells to the tumor (34).

While the immunohistochemical detection of EGFR expression did not prove to be decisive in determining the clinical response, promising data has been generated in support of using the EGFR gene copy number as a predictive biomarker for EGFR-targeted therapy (37). Nevertheless, in order to achieve definitive proof and to facilitate the development of standardized testing modalities further investigation is required. In NSCLC, EGFR is targeted primarily using the small molecule inhibitors gefitinib and erlotinib. In contrast to colorectal cancer, mutations within the EGFR in NSCLC are common and mutational testing is recommended for all NSCLC cases (38). The application of mutation-specific antibodies for this purpose is discussed below. A further distinguishing feature regarding the testing of EGFR in colorectal cancer and NSCLC is that in the latter, IHC positivity or high EGFR gene copy numbers showed no conclusive correlation with treatment response (38).

V-KIT HARDY-ZUCKERMAN 4 FELINE SARCOMA VIRAL ONCOGENE HOMOLOG

Immunohistochemistry provides an excellent tool for the differential diagnosis of gastrointestinal stromal tumors (GIST). This is largely due to the fact that greater than 85% of GIST test positive for v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT), in contrast to the negative result generated by most other mesenchymal tumors (39, 40). Imatinib mesylate (Gleevec, Novartis, Basel, Switzerland) is a specific tyrosine kinase inhibitor that exhibits high therapeutic activity in patients with chronic myeloid leukemia by targeting the fusion protein BCR-ABL (41). Additional targets of imatinib mesylate include the platelet-derived growth factor receptor (PDGFR) and KIT. For patients with unresectable or metastatic GIST, a positive immunohistochemical staining for KIT was initially required as an entry criteria into clinical trials investigating the efficacy of imatinib mesylate (42, 43). Significant clinical responses were recorded and this revolutionized the management of advanced GIST, a malignancy that had previously failed to respond to conventional chemotherapy. Activating mutations within the KIT gene and, to a lesser degree, PDGFR, are commonly found in patients with GIST and depending on their location within the coding region of the respective gene, they are highly correlated with the likelihood of a response to imatinib mesylate treatment (44, 45). That notwithstanding, since a small percentage of patients with GIST do not express detectable levels of KIT or do not harbor mutations within KIT/PDGFR (44–46) IHC or mutational analysis should not be used to deny treatment with imatinib mesylate since these patients may still be sensitive to this therapy.

ANAPLASTIC LYMPHOMA KINASE REARRANGEMENTS

In addition to EGFR, the anaplastic lymphoma kinase (ALK) represents a second therapeutic target in NSCLC. Chromosomal rearrangements involving the associated gene have been detected in approximately 5% of cases, most frequently resulting in the fusion to echinoderm microtubule-associated protein-like 4 (EML4) and the constitutive expression of a chimeric tyrosine kinase protein (47, 48). Second-line treatment of NSCLC patients with confirmed ALK rearrangements, using the small molecule inhibitor crizotinib, has recently been shown to significantly prolong progression-free survival compared to standard chemotherapy (49). The current "gold standard" for ALK rearrangement testing is dual-color break-apart FISH. However, interpretation of test results may be challenging since EML4 and ALK are located on the same chromosome, resulting in limited separation of the 5′ and 3′ probes. To define a positive test result, only signals separated by more than two signal diameters and/or single 3′ signals (correlating to the ALK kinase domain) should be counted. In addition, at least 50 cells should be reviewed with a positive signal detectable in at least 15% (50). The immunohistochemical detection of ALK has been considered as an attractive addition to routine FISH testing. Since ALK is not expressed in lung tissue unless driven by promoter rearrangement, a good correlation between IHC and FISH results and low IHC background staining have been reported. In addition, a number of studies confirm that IHC-negative cases are almost exclusively negative in FISH analysis and therefore indicate that IHC could be applicable as a quick

and cost-effective screening tool for ALK rearrangements (51–54). FISH reflex testing for all IHC positive cases has been proposed, somewhat similar to the evaluation strategy for HER2 (55). Interestingly, the percentage of ALK rearrangement positive cells during FISH evaluation did not significantly correlate with the response to crizotinib (56). Nonetheless, comprehensive data on the correlation between the intensity of ALK staining as determined by IHC and treatment response is still lacking.

MUTATION-SPECIFIC ANTIBODIES

The selection of patients for a targeted cancer treatment frequently relies on the detection of specific gene mutations. The routinely applied techniques are generally based on the isolation of chromosomal DNA from fresh, frozen or FFPE material and analysis can involve various techniques such as mutation-specific real-time PCR, direct sequencing, mass spectrometry, mismatch ligation assays, high-resolution melting curve assays, or denaturating high-performance liquid chromatography, among others. A common drawback relates to the fact that information on tissue morphology is lost and also that "contamination" of tumor material with normal cells may hamper detection or obscure the results. In addition, increased demands on sample size and quality and extra requirements regarding technology and expertise, associated with higher cost and expenditure of time, frequently apply. The development of mutation-specific antibodies and their application in routine IHC may provide a convenient addition to DNA-based profiling techniques.

BRAF V600E

The v-raf murine sarcoma viral oncogene homolog B1 (BRAF) represents an outstanding target for the development of a mutation-specific antibody. Mutations of the associated gene occur in a range of human malignancies including cutaneous melanoma, colorectal cancer, NSCLC, papillary thyroid cancer, and hairy-cell leukemia (57–59). By far, the most common BRAF mutation results in the substitution of valine for glutamic acid at position 600 (V600E), leading to the constitutive activation of the protein's kinase domain. In human cutaneous melanoma, mutated BRAF has been detected in 40–50% of cases, with up 90% of these alterations concerning the V-E substitution at codon 600 (60). Vemurafenib and dabrafenib are two potent small molecule inhibitor drugs that specifically target BRAF V600E and have demonstrated remarkable response rates in metastatic melanoma patients (61, 62). Mutational testing of the patient tumor material is required before commencement of treatment and is to date commonly based on the detection of genomic alterations. As an addition to DNA testing, Capper et al. recently proposed a mutation-specific antibody for the detection of BRAF V600E in FFPE tissue specimens by means of IHC (**Figure 1**) (63). The results obtained from using this antibody to determine the BRAF mutational status in melanoma and thyroid cancer samples were identical to those achieved following DNA sequencing-based profiling. These results have since been substantiated by numerous studies with similarly high levels of specificity and sensitivity (up to 100%, respectively) (64–68).

In samples where the number of tumor cells is low, IHC-based BRAF testing was suggested to be more sensitive than direct DNA sequencing or high-resolution melting curve analysis (64, 65). In addition, IHC-based staining results showed low inter-observer variability (68). Nevertheless, despite the benefits of using IHC-based methods for determining the presence/absence of mutations within the BRAF gene, the presence of (non-specific) strong,

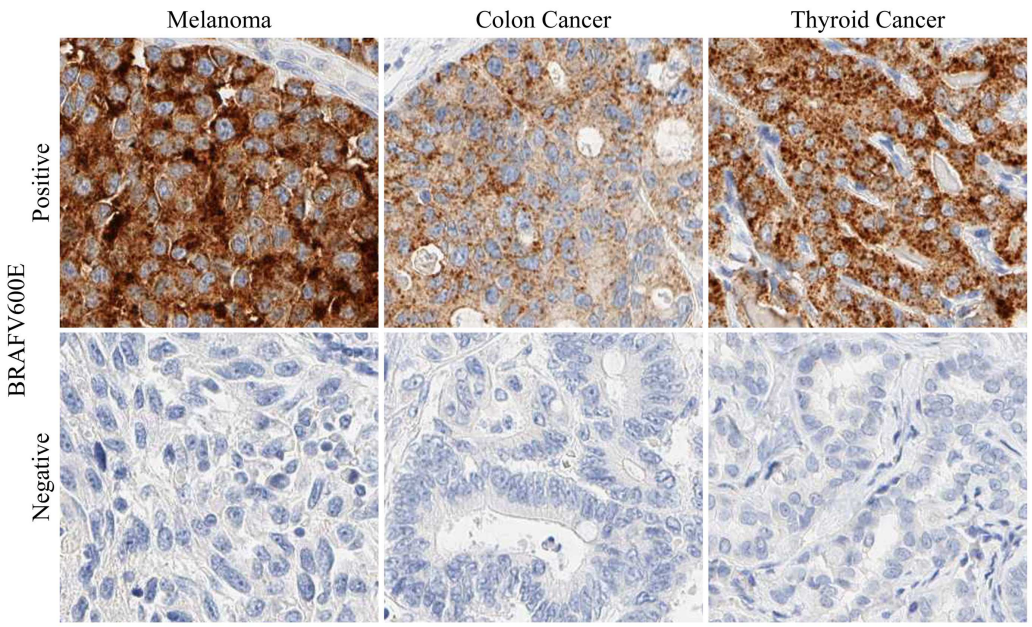

FIGURE 1 | BRAFV600E mutation-specific antibody staining. Immunohistochemical staining examples of the BRAFV600E mutation-specific antibody VE1 are presented for a BRAFV600E-positive and a BRAFV600E-negative case of melanoma, colon cancer, and thyroid cancer, respectively. BRAFV600E-positive staining is generally detected as a granular, cytoplasmic signal that can easily be distinguished from BRAFV600E-negative cases. In the presented example images, 3,3′-Diaminobenzidine (DAB) was used as a chromogen.

nuclear staining may complicate the assessment of staining results. In addition, false-negative IHC results may occur due to unsuitable or incomplete tissue fixation, the presence of necrotic or pre-necrotic tissue areas or low levels of total protein expression (63, 64). The latter may be controlled for through the use of an additional antibody capable of detecting total BRAF protein expression. With regard to the intra-tumor heterogeneity of BRAF V600E, only few cases with non-homogenous expression have been observed following immunohistochemical detection (67, 69). These results are in direct contrast to previous reports describing significant variability of BRAF mutational status among individual cells within a tumor and warrant further investigation (70, 71). Regarding the possible relationship between BRAF V600E protein expression and clinical outcome, no significant correlation was seen between the percentage of BRAF V600E positive tumor cells and the response to treatment with either dabrafenib or vemurafenib (72). Similarly, the total intensity of mutation-specific antibody staining did not significantly correlate with patient outcome.

EGFR L858R AND E746_A750del

Epidermal growth factor receptor mutations have been detected in 2–17% of NSCLC patients from Europe and the United States, however, the mutational frequency increases to 30% when analyzing cases from East Asia (73–75). Specific mutations, in particular those affecting the EGFR kinase domain, have been associated with response to gefitinib and erlotinib treatment (76, 77). The two most common types of EGFR mutations are in-frame deletions of exon 19 and a leucine to arginine substitution at codon 858 (L858R) in exon 21. Taken together, alterations at these sites account for up to 90% of all EGFR mutations (78). Exon 19 deletions may affect a varying number of nucleotides. For example, E746_A750del results in a five amino acid deletion in the corresponding protein and is the most common deletion detected, occurring in approximately 70% of cases (79). The application of mutation-specific antibodies designed to target L858R and the E746_A750del modification, have yielded varying levels of detection specificity and sensitivity. For L858R, several studies reported sensitivity values in the range of 70–100% and specificities exceeding 95% (79–83). While these results were promising, sensitivities as low as 36 and 40% have also been described for the same antibody clone (84, 85). Similarly, regarding the E746_A750del-specific antibody, the same studies published sensitivity and specificity values of 40–100% and 95–100%, respectively. The possible sources of variation are numerous and include the application of different scoring systems, discrepancy between the definitions of positivity/negativity, different DNA-based reference techniques, different tissue fixation methods, and the types of specimens analyzed. While initial attempts to determine optimal tissue preparation and staining evaluation have been presented (86), additional steps toward a standardized protocol for the detection of EGFR mutations using IHC should be undertaken.

The overall high levels of specificity associated with IHC-based EGFR-mutational testing imply that IHC may be suitable as a prescreening tool for the identification of NSCLC patients that are eligible for EGFR-inhibitor treatment. Since a number of mutations are not currently detectable by antibody-based profiling, the additional testing of IHC-negative cases using direct DNA sequencing or similar assays is necessary (79, 81). The applicability of IHC in predicting response to EGFR-targeted therapy remains controversial. Confirming the importance of EGFR-mutational status for treatment response, positive IHC staining has been associated with longer progression-free survival compared to IHC-negative or -equivocal cases (83, 87). In addition, high mutant EGFR expression (as defined by the sum of scores for fraction and intensity) was significantly related to elevated progression-free survival but not overall survival (88) and a fraction of positive tumor cells exceeding 50% of all cells predicted better response to EGFR inhibition treatment in univariate but not multivariate analysis (89). Despite the aforementioned results, a study by Kato and co-workers could not detect a significant correlation between IHC staining and treatment response or survival (82). Shortcomings in the significance of IHC-based detection methods in predicting survival benefit, in particular when compared to DNA-based techniques, may occur due to the limited mutation spectrum detected via IHC. Furthermore, EGFR-mutation-specific antibodies have been shown to occasionally detect mutations associated with EGFR-inhibitor resistance via mechanisms that are not yet fully understood (84).

ALTERNATIVE PROTEIN BINDERS

In situ affinity-based detection of proteins remains one of the best sources of information about either the healthy status of an individual tissue or potential pathological changes, and is thus applicable within several medical settings. Molecular imaging allows for the early detection and classification of many human diseases and, when specific, permits improved, target-directed therapies. Molecules generated through immunization such as polyclonal, monospecific polyclonal, and monoclonal antibodies continue to be the best established and most widely used binders in diagnostics (90, 91). Methods for the detection of proteins based on antibody recognition often encounter problems due to poor selectivity and/or sensitivity (92). Poorly characterized antibodies and/or insufficient quality control often render them as unsuitable for demanding applications such as companion diagnostics (93). Commercially available antibodies frequently perform very differently within various laboratories and often do not perform as advertised, thus raising doubts regarding their reliability when incorporated into assays requiring high specificity (94). Antibodies can be biochemically and physiologically modified and use of their derivatives, such as single chain variable fragments (scFv) or Fab fragments, may result in the improved detection of a wide range of target molecules (**Figure 2**) (95). Currently only a few antibodies and recombinant proteins are used within clinical settings, largely due to the reasons outlined above. Recombinant binders that are generated in immune-free, *in vitro*-based approaches, hold the potential of taking priority over conventional antibodies (96). Stability, specificity, ease of manipulation, low cost, high throughput, and reproducibility of production are some of the advantages that make novel scaffold molecules highly desirable (97). Alternative binders are promising molecules for novel approaches in individualized medicine (**Figure 2**). They can serve as personalized molecular imaging tools for *in vivo*, live diagnostics of the changes occurring in the expression of markers following treatment (95).

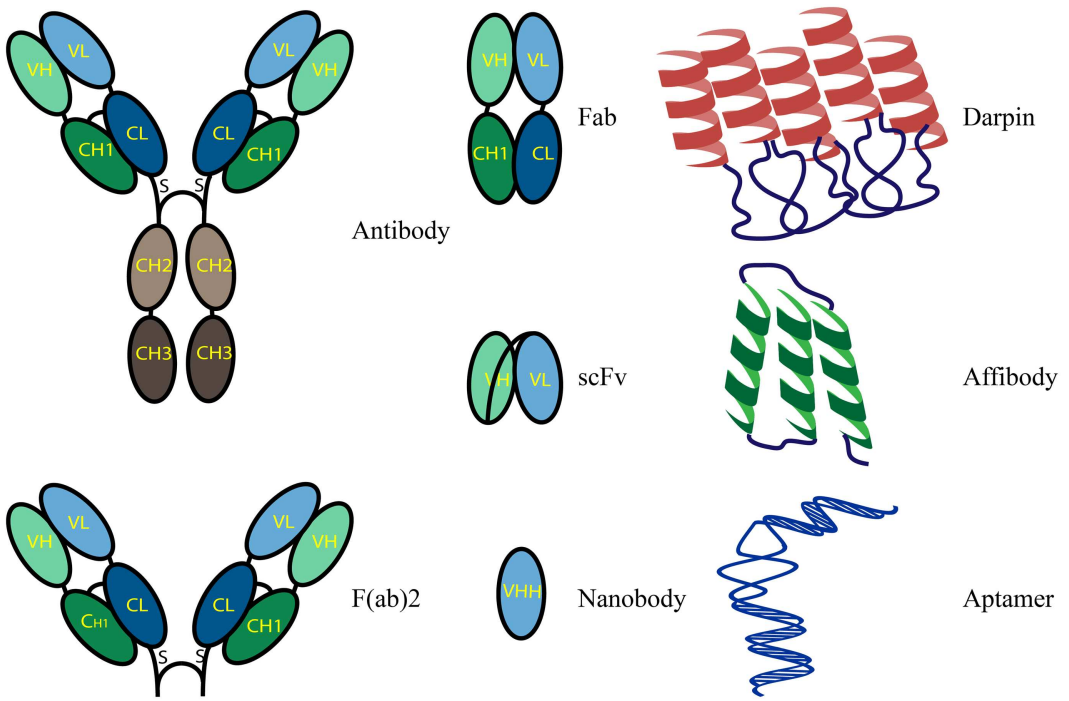

FIGURE 2 | Schematic representation of binder formats.

RECOMBINANT ANTIBODY FRAGMENTS

Antibody fragments are generated in order to obtain binders with improved characteristics, compared to conventional antibodies, but similar functional and recognition properties. Fab and F(ab)$_2$ fragments are antigen-binding fragments generated after proteolysis of full length antibodies. Single chain variable fragments (scFvs) are smaller than Fab fragments and are composed of genetically linked light and heavy chains containing variable regions. These molecules can also exist as dimers (98). Another class of antibody-derived antigen-binding molecules, termed nanobodies, are small, single-domain polypeptides derived from the variable part of the heavy chain (VHH) of light-chain deficient antibodies that were originally discovered in camelids (camels, llamas) (99, 100). Their reduced size, greater stability and solubility, and antibody-like binding characteristics make nanobodies ideal for use in the targeting and imaging of antigens in live cells, protein precipitation *in vivo*, and targeted enzymes modulations (101–103). As a result of their smaller size (only 15 kDa), nanobodies can bind to epitopes that are hidden or shielded and reach affinities within the range of nanomolar to picomolar. Nanobodies are highly specific for their targets and have no known cross-reactivity to structurally related proteins, which makes them excellent tools for targeting kinases and tyrosine phosphatases. In addition, nanobodies have technological advantages that render them superior to conventional antibodies. They are easily modified to avoid chemically reactive groups such a primary amines or to alter the primary amine number to allow more selective and controlled chemical conjugation. The selection of binders for certain applications is usually based on their performance in experimental settings and depends on their preference for particular epitopes and the accessibility of binding sites.

AFFIBODY MOLECULES

Affibody molecules are small (∼7 kDa), alpha-helical *Z*-domain of Staphylococcal protein A, immune-independent affinity molecules that target a wide range of proteins (104, 105). They can be produced in functional form both via recombinant expression in *Escherichia coli* or peptide synthesis. They possess picomolar affinities, are highly soluble and stable. In addition, they are cysteine-free which prevents non-specific binding events when applied to tissues. Furthermore, the lack of cysteine provides an opportunity for site-specific labeling through the introduction of unique cysteine molecules. High affinity affibodies were engineered against targets such as the IL2 receptor, Alzheimer's amyloid-beta peptide or EGFR (106–108). They have been used in various types of experiments and are intended for both *in vivo* and *in vitro* imaging and also therapeutic applications including the detection of HER2 within different experimental settings (109, 110).

DESIGNED ANKYRIN REPEAT PROTEINS

Designed ankyrin repeat proteins (DARPins) are potent alternatives to conventional antibodies. They detect antigens with high specificity and picomolar affinity, are independent of target immunogenicity and possess attractive molecular properties such as small size and high stability (111). They are synthetic, non-immunoglobulin binding proteins that form scaffolds containing

tandem repeats of an elementary, structural motif, typically composed of 33 amino acid residues folded into a β-turn followed by two antiparallel α-helices. A single protein may contain up to 29 repeats of this motif. The production of DARPins does not require the use of animals at any step, therefore permitting the large scale, parallel production of variable binders. DARPins are correctly folded in both prokaryotes and eukaryotes, due to the absence of disulfide bonds, which enables their use in a variety of functional assays (112). They can easily be genetically modified to form fusion proteins and site-specifically targeted for chemical conjugation. DARPins against a wide range of protein targets, including extracellular, intracellular, and membrane proteins, were generated with high yield from synthetic libraries and successfully used as replacements for conventional antibodies. One such example is the DARPins generated against HER2; these displayed higher specificity and similar sensitivity when compared to FDA-approved antibodies for the *in situ* identification of HER2 expression status in FFPE breast cancer tissue (113). DARPins can easily be made functional for use in various biomedical applications through the introduction of site-specific, clickable modifications. Such alterations do not affect their physical properties (114).

APTAMERS

Aptamers are a class of small, synthetic, self-folding, and single-stranded RNA or DNA molecules that form secondary and tertiary structures and specifically bind to proteins, small molecules, or other cellular targets such as nucleic acids (115, 116). They are comparable to antibodies in terms of their target recognition capabilities, their binding affinities, and the diversity of applications that they can be used in; however, they possess numerous significant characteristics that render them advantageous over their protein equivalents. Aptamers are highly specific, non-immunogenic, redox-insensitive, and temperature- and pH-tolerant. In addition, they do not have hydrophobic cores which are usual in proteins and, therefore, they do not aggregate. In order to select aptamers, information on protein conformation is not required, a feature which can be useful for screening for unidentified disease biomarkers. Aptamers can be generated through cell-based aptamer selection that utilizes differences between the molecular signatures of any two different cell types. The selected aptamers selectively bind to an unknown protein within one cell type only, are cross-linked to their targets and once the complex is purified the targets can be analyzed by mass spectrometry (117). Therefore, the cell-based selection of aptamer molecules has great potential for the development of specific probes suitable for biomarker discovery and companion diagnostics development. Since chemical synthesis is a process that is well defined and highly reproducible, the production of aptamers can easily be scaled up. They can be synthesized with specific, custom tailored functional groups attached to 5′ or 3′ termini which creates an easy approach to conjugation and multiplexing *in situ* assays. Furthermore, aptamer conjugations do not generally alter their binding affinity. With advances in imaging techniques, aptamers are already considered as prospective reagents for *in situ* targeting. To date, several aptamers have been developed against important clinical targets such as PDGF, von Willebrand factor (vWF), E-selectin, vascular endothelial growth factor (VEGF), and prostate specific membrane antigen (PSMA) and their applicability within a clinical setting is currently being investigated (118–121).

IN SITU PROXIMITY LIGATION ASSAY

Using traditional IHC techniques, the level of protein expression can easily be determined. However, the functional status of a cell cannot be evaluated by the level of expressed protein alone. The activity of signaling pathways, as assessed by the analysis of post-translation modifications (PTMs) and protein interactions, needs to be determined and taken into consideration (122). Cancer does not consist of a homologous mass of cells but of complex, heterogeneous cell populations that are affected by interactions with each other and the surrounding environment. Therefore, the analysis of cancer tissue at single cell resolution provides a much better understanding of the differences in signaling status and activity (123). *In situ* PLA enables a localized and specific detection by utilizing oligonucleotide-conjugated antibodies to determine the proximity between one or more targeted epitopes. This makes it a suitable method for detecting molecular events in cells and tissue, for example the status of a signaling pathway. The use of two independent binders and the additional requirement of proximity for reporting enable the specific detection of proteins, protein–protein interactions, and PTMs (124). Proximity ligation converts the recognition of a protein, protein complex, or PTM by two or more antibodies into an amplifiable, circular DNA molecule (**Figure 3**). Upon proximal binding of a pair of oligonucleotide-conjugated antibodies (PLA probes), the oligonucleotides guide the formation of a circle after applying two additional, single-stranded DNA molecules. This circular DNA molecule is then ligated and amplified by phi29 polymerase within a rolling-circle amplification (RCA) reaction, resulting in a localized, concatameric product. The latter is visualized by hybridization of detection oligonucleotides labeled with fluorophores or horse radish peroxidase (**Figure 3**) (125). Due to the environment of fixed cells and tissue the amplification product will collapse into a bundle with a diameter of approximately 1 μM (126) that can then be visualized as a bright dot that is quantifiable and easily distinguished from the background (127).

Heterogeneity within a sample increases the demands on the dynamic range of a method in order to allow for the detection of both abundant and scarce targets within the same sample. *In situ* PLA uses an amplifiable DNA circle as a reporter molecule and by using reagents that give rise to three variants of the reporter DNA circles and adding them at decreasing concentrations the dynamic range of the PLA is increased. By labeling the circles with different fluorophores, the readout can be adjusted to the fluorophore whose concentration gives rise to quantifiable and easily distinguishable signals. In a heterogeneous sample, different readout fluorophores can be used for different parts of the sample, enabling the detection of a target that varies greatly within the sample without the risk of signal saturation. As the size of a patient sample is often limited, this approach reduces the need to optimize the binder concentration and enables the evaluation of patient samples where knowledge on the expected results is limited (128).

Multiplex *in situ* PLA permits the parallel analyses of multiple protein complexes involved in signaling pathways directly in tissue and cells thus making it possible to compare levels of

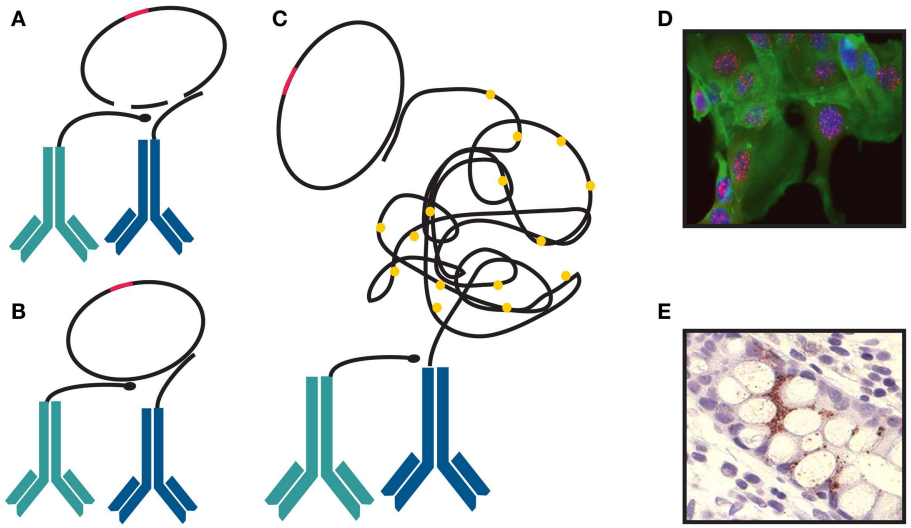

FIGURE 3 | Proximity ligation assay. **(A)** Two probes stay in close proximity by binding to a protein or two proteins present in one complex. **(B)** They are joined and circularized by DNA ligation upon introduction of linear connector oligonucleotides. After ligation, rolling-circle amplification (RCA) is initiated. One of the proximity probes is used as a primer. **(C)** The single-stranded RCA products are hybridized with labeled detection oligonucleotide complementary to a multiplied motif in the sequence of the RCA product. The detection oligonucleotide can be labeled with fluorophore **(D)** or a horse radish peroxidase **(E)**.

protein complexes between individual cells and also providing information regarding the spatial distributions of these complexes. A tag-specific sequence within the PLA probe targeting a protein gives rise to a DNA circular molecule that carries information on the identity of the target protein. The amplified tags in the RCA products can then be visualized using oligonucleotides labeled with different fluorophores, to uniquely recognize the tag sequences corresponding to a certain target (129). *In situ* PLA has been shown to provide valuable information about the status of signaling pathways by detecting molecular events such as dimerizations, the formation of protein complexes and PTMs. It enables the detection of activity at different levels within a signaling pathway, thereby enabling specific aberrations to be pinpointed. *In situ* PLA has been utilized in studies investigating both EGFR dimerization and receptor activation, which has been proposed to play a crucial role during tumor progression, and also the development of drug resistance. Dimerization and aberrant activity has been shown to be independent of EGFR expression, explaining why the deregulated expression of the EGFR in several types of human malignancies was shown to have limited value as a prognostic or diagnostic marker. Receptor dimerization events detected by mutation-specific PLA appeared to be more suitable for the selection of patients for EGFR-targeted treatment (130).

Similarly, the overexpression of human epidermal growth factor receptors (HERs) has been linked to poor prognosis in patients with early breast cancer. Dimers containing the HER2 isoform were shown to be more stable and have prolonged active signaling compared to HER2 deficient dimers. Elevated levels of HER2-HER2 and HER2-HER3 complexes detected by PLA showed a significant association with decreased recurrent-free survival and a reduction in overall survival of breast cancer patients, proving that PLA and the detection of cellular signaling processes can be successfully implemented in studies on prognostic markers in clinical specimens (131). Through the application of *in situ* PLA, it is now possible to screen for the effects of a drug treatment on intracellular signaling, providing information on the specific level of signaling pathways. Being able to study primary cell lines and patient tissue sample gives valuable information of the signaling status within a specific tumor and allows to predict the response to a certain therapy (132). PLA technologies have been used to address a variety of biomedical problems and demonstrated the potential to address some difficulties, both concerning the validation of biomarkers and the applicability for clinical diagnostics. Implementing PLA techniques as an alternative to IHC in everyday laboratory practice allows for a more precise and quantitative evaluation of antibody performance characteristics and their suitability for an anticipated analytical use. Application of the PLA technique provides an opportunity to develop a high-quality procedure for *in situ* detection of proteins and signaling pathways in companion diagnostics. This will offer the medical industry powerful, universally applicable tools for clinical research and routine diagnostics.

CONCLUSION

Affinity proteomics for the analysis of proteins as companion diagnostics requires access to reagents that can be used in specific detection reactions. The comprehensive validation and improvement of existing and newly generated antibodies to obtain well characterized, high-quality, and well-controlled resources as tools for large scale studies of the human proteome in health and disease is a widely acknowledged demand. In many routine clinical, diagnostic, and life science applications, antibodies have proven to be the reagents of choice. IHC is routinely performed in the majority of clinical laboratories and widely acknowledged as superior

to other analysis techniques regarding time- and cost-effective application. In selected cases, IHC and mutation-specific antibodies may even provide an attractive alternative to DNA-based testing methods. As a highly valuable resource documenting the availability and identification of novel biomarker candidates, the Human Protein Atlas[2] project has generated antibodies targeting proteins from over 15,000 genes, corresponding to about 75% of all human protein-coding genes (133, 134). All antibodies are routinely subjected to a series of validation steps, including protein arrays, western blots, and immunofluorescence and used to assess protein expression patterns in a broad spectrum of normal and cancer tissues through application of IHC. Nevertheless, efforts to detect proteins when high specificity is required often fail. Hence, there is a strong need for better methods and reagents for assessing protein expression in tissues as means of companion diagnostics and alternative binders or PLA in combination with existing antibodies represent promising candidate alternatives.

ACKNOWLEDGMENTS

This work was supported by grants from the Knut and Alice Wallenberg Foundation, from the Swedish Cancer Society and from the European Union Seventh Framework Program under grant agreement 241481 (Affinomics).

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[2]www.proteinatlas.org

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 September 2013; paper pending published: 15 October 2013; accepted: 17 October 2013; published online: 31 October 2013.

*Citation: Gremel G, Grannas K, Sutton LA, Pontén F and Zieba A (2013) In situ protein detection for companion diagnostics. Front. Oncol. **3**:271. doi: 10.3389/fonc.2013.00271*

This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology.

frontiers in ONCOLOGY

REVIEW ARTICLE
published: 17 April 2014
doi: 10.3389/fonc.2014.00078

Navigating the rapids: the development of regulated next-generation sequencing-based clinical trial assays and companion diagnostics

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Over the past decade, next-generation sequencing (NGS) technology has experienced meteoric growth in the aspects of platform, technology, and supporting bioinformatics development allowing its widespread and rapid uptake in research settings. More recently, NGS-based genomic data have been exploited to better understand disease development and patient characteristics that influence response to a given therapeutic intervention. Cancer, as a disease characterized by and driven by the tumor genetic landscape, is particularly amenable to NGS-based diagnostic (Dx) approaches. NGS-based technologies are particularly well suited to studying cancer disease development, progression and emergence of resistance, all key factors in the development of next-generation cancer Dxs. Yet, to achieve the promise of NGS-based patient treatment, drug developers will need to overcome a number of operational, technical, regulatory, and strategic challenges. Here, we provide a succinct overview of the state of the clinical NGS field in terms of the available clinically targeted platforms and sequencing technologies. We discuss the various operational and practical aspects of clinical NGS testing that will facilitate or limit the uptake of such assays in routine clinical care. We examine the current strategies for analytical validation and Food and Drug Administration (FDA)-approval of NGS-based assays and ongoing efforts to standardize clinical NGS and build quality control standards for the same. The rapidly evolving companion diagnostic (CDx) landscape for NGS-based assays will be reviewed, highlighting the key areas of concern and suggesting strategies to mitigate risk. The review will conclude with a series of strategic questions that face drug developers and a discussion of the likely future course of NGS-based CDx development efforts.

Keywords: companion diagnostics, disruptive technology, precision medicine, next-generation sequencing, clinical next-generation sequencing, molecular diagnostics, drug development strategy, mutation detection methods

INTRODUCTION

The concept of personalized medicine relies heavily on access to information on an individual's unique genetic characteristics to tailor therapy. However, the current paradigm of regulated molecular diagnostic (Dx) testing, in which individual Food and Drug Administration (FDA)-cleared Dx tests are employed to detect mutations in a single gene, sits uneasily in this framework of personalized medicine (1, 2). The advent of clinical next-generation sequencing (NGS) has begun to provide to the clinic a more expansive insight into genetic mutations in a broader set of genes, usually drawn from pathways implicated in and actionable by current therapeutics or by promising drug candidates in development (3). NGS-based diagnosis is specially promising for diseases that have a highly complex and heterogeneous genetic composition. The field of oncology is therefore very well positioned to benefit greatly from such an approach (4, 5). Since NGS-based technology permits a more complete view into a tumor's genetic composition, it is easy to foresee that treatment paradigms must

Abbreviations: ABRF, Association of Biomolecular Resource Facilities; ACMG, American College of Medical Genetics and Genomics; BRAF, v-Raf murine sarcoma viral oncogene homolog B; CAP, College of American Pathologists; CDER, Center for Drug Evaluation and Research; CDRH, Center for Devices and Radiological Health; CDx, companion diagnostic; CE Conformité Européenne, Conformity Marking for Relevant European Council Directives; CFTR, cystic fibrosis transmembrane conductance regulator; CLIA, Clinical Lab Improvement Amendment; CRO, contract research organization; CTA, clinical trial assay; ddNTPs, dideoxynucleotide triphosphates; DNA, deoxyribonucleic acid; dNTPs, deoxynucleotide triphosphates; Dx, diagnostic; EGFR, epidermal growth factor receptor gene; EMR, electronic medical records; ePCR, emulsion PCR; FDA, United States Food and Drug Administration; FFPE, formalin fixed paraffin embedded; gDNA, genomic DNA; IHC, immunohistochemistry; ISPs, ion sphere particles; IUO, investigational use only; IVD, *in vitro* diagnostic; KRAS, Kirsten rat sarcoma viral oncogene homolog gene; LDT, lab-developed test; MAQC, microarray quality control; NCI, National Cancer Institute; NGS, next-generation sequencing, NIST, National Institute of Standards and Technology; NTC, no template control; PacBio, Pacific Biosciences; PGM, Personal Genome Machine; PMA, pre-market approval; PT, proficiency testing; QC, quality control; qPCR, quantitative polymerase chain reaction; RNA, ribonucleic acid; RNA-Seq, RNA sequencing; RUO, research usage only; SMRT, single molecule real time sequencing; SNV, single nucleotide variant; TAT, turnaround time; UTR, untranslated region; VCF, variant calling file; ZMW, zero-mode waveguides.

change accordingly to allow treatment based on the molecular pathological fingerprint of the individual. As a result, the question is not technological ("Can it be done?"), but rather practical ("How can NGS technology be developed into a mainstream multi-gene or multi-transcript Dx fingerprint?") and regulative ("What are the barriers that must be overcome for this disruptive technology be approved as a general companion diagnostic (CDx) device for multiple therapeutics?"). It is clear the scientific community is rapidly embracing the technology as NGS-based tests are being employed across multiple disease areas, including oncological, metabolic, cardiovascular and neurosensory disorders, and in prenatal diagnoses (6–10) where genetic components are defined. As of late 2013, several dozen clinical labs offer over 50 different laboratory-developed tests (LDTs) using NGS (11). These tests are offered as single-gene assays or multi-gene or multi-transcript panels. Commercially available NGS-based cancer panels are already being used in clinical practice and as clinical trial assays (CTAs) to guide patients to most appropriate experimental treatment (8, 12, 13). Nonetheless, there are no FDA-approved NGS CDxs available today and there are significant challenges in developing such tests. We compare developing NGS-based Dx to navigating the rapids, an exercise full of challenges, continuously changing technologies, policies, and regulations as the field develops at a rapid pace, and yet the promise of personalized medicine is within reach and closer than ever before.

CURRENT PARADIGM IS UNSUSTAINABLE

Precision medicine has been defined as identifying the right drug, for the right patient, at the right dose, at the right time (14). Intrinsic to identifying the right patient is a Dx device. If it is linked to a specific therapeutic and if the test is required for the safe and effective use of the drug, then Dx device is termed a CDx. The current testing paradigm for precision medicine links a specific drug to the Dx (15, 16) and can be summarized as "one-drug/one-gene Dx." This is abundantly illustrated for FDA-approved Dxs, such as the one-gene tests approved for mutations in *EGFR*, *KRAS*, and *BRAF*. Yet, it is equally clear that the current paradigm is not sustainable (17, 18). First, cancer is an exceedingly complex molecular and epigenomic disorder, resulting from perhaps hundreds of different molecular defects, including somatic mutations, gene expression changes, and genome rearrangements. Furthermore, tumorigenesis and tumor progression are driven by altered gene regulation networks that are not always tractable to a clear and defined somatic mutation (19). Recent results from clinical studies support the emerging concept of the "mutation signature" or spectrum of correlated mutations in cancer (20, 21), which postulates that the combination of mutations present is more predictive of the response to treatment than individual gene mutation status. Thus, to ensure their patients are offered the best possible treatment, physicians will want to examine the tumor's whole cancer genome, both somatic mutation and transcriptional changes, to identify the most personalized therapy, and they will do so whether or not there is a FDA-sanctioned Dx for a particular drug. Instead, they will use LDTs, which the FDA believes should be regulated as *in vitro* diagnostics (IVDs) (75 FR 34463, 2010). Thus, the current situation is untenable since it is only a matter of time before more comprehensive tests will routinely be used to diagnose a patient's tumor. Second, not only do physicians need more molecular information, but patients want it too. In this age of internet medicine, many patients are well-informed and strongly advocate for more comprehensive testing, even to the point of paying for it themselves in order to get a more complete picture of their cancer (22). Their reasoning that more information is better is hard to argue against. Hospitals and for-profit companies have developed tests to meet this need, and advertisements for comprehensive genomic tumor assessment on television, radio, and internet are not uncommon. Furthermore, patients considering a clinical trial at a major hospital are beginning to expect molecular characterization of their tumor as a *quid pro quo* for participation in the clinical study. A third, more practical, reason why the current model is not sustainable is the limitation of tissue. A Dx tumor specimen block can only be sectioned into a limited number of sections. Sample is limiting and tests are not currently multiplexed; separate slides are usually required for different immunohistochemical (IHC)-, ribonucleic acid (RNA)-, or deoxyribonucleic acid (DNA)-based tests. In most cases, there is simply not enough material to test for every gene mutation that is available, and therefore a more efficient use of the patient's specimen is needed. For these reasons, it is clear the "one-drug/one-gene Dx" paradigm is unsustainable and that the drive toward precision medicine is changing clinical practice, and as it does, it will change the clinical testing paradigm for cancer treatment decisions.

DISRUPTIVE SHIFT

Next-generation sequencing is a classic disruptive technology (23). It may even change the way precision medicines are developed (24). Although these changes will impact the healthcare community and their patients, in this section we will only focus on the potential impact on drug developers and manufacturers of Dx tests. The crux of these changes is the shift from a "one-drug/one-gene Dx" model to a "multi-gene Dx/many drugs" paradigm (25, 26). An oversimplification of the interaction between the drug developer and the Dx company can be summarized as: the drug company develops a promising drug and discovers late in development that a Dx is needed to identify the appropriate patient population. Then it works with the Dx company to develop the test to detect and/or quantify the specific biomarker, and they are both tested in pivotal trials. Thus, the drug drives the device development. The use of a multi-gene or multi-transcript panel has the potential to change that. Instead of a single drug developer partnering to develop a single Dx test, what may happen is that the device manufacturer may design an assay able to detect a myriad of RNA or DNA biomarkers. That is, the device manufacturer may drive content on the device and may proactively seek FDA-clearance independent of a partnership with a drug maker. The implication of this disruptive shift is a set of challenges that will be discussed in a later section.

PRIMER ON NGS PLATFORMS

Several firms have developed small benchtop NGS sequencers for the clinical Dxs market. The current leading platforms are the MiSeq from Illumina, Inc. and the Personal Genome Machine (PGM) from Life Technologies, Inc., which together comprise >85% of market as of early 2014 (Bloomberg Businessweek,

January 2014). The recent agreement between Roche Molecular Diagnostics and Pacific Biosciences (PacBio) heralds the entry of the latter into the Dx arena. Qiagen has announced that it will release its benchtop GeneReader™ NGS platform in 2014. Key factors that influence clinical labs' adoption of a particular platform include sequencing quality, turnaround time (TAT), cost per sample, optimal ease of use for the operator, and sample multiplexing capability (recognizing that multiplexing is likely required to reduce cost). We provide a brief overview of the main clinical NGS technology platforms here and refer the reader to exhaustive reviews on NGS technology and instrumentation advances for further details on each (27–30).

ION TORRENT

Life Technologies' Ion Torrent semiconductor sequencing technology, which made its debut in 2011, is based on a sequencing-by-synthesis approach in which individual templated DNA molecules positioned in microwells on a semiconductor chip are sequentially incubated with each of the four deoxynucleotide triphosphates (dNTPs) to support DNA strand polymerization (31). Only the dNTP complementary to the template is incorporated at the end of each template strand. As each dNTP is incorporated, a proton is released, which acts as an indicator of base incorporation and the number of bases incorporated consecutively. The resulting pH changes are recorded as voltage changes that convey the sequence of bases for the flow. Advantages of this technology include optics-free readout, low input DNA requirement (which is critical for clinical practice), and longer read length with accurate base calling (32).

ILLUMINA

The Illumina technology also utilizes a sequencing-by-synthesis approach with bridge amplification (27). Clonally amplified DNA templates are immobilized to an acrylamide coating on the surface of a glass flowcell that serves as the reaction and sequencing substrate. Fluorescently labeled reversible-terminator dideoxynucleotide triphosphates (ddNTPs) are added one base at time in this sequencing technology. After the addition of each nucleotide, the clusters in the flowcell are imaged to determine which fluorescent dye was incorporated. In its current manifestation, Illumina's greatest strength is the easier workflow of the amplicon library preparation and reduced hands-on time as compared to other platforms. Data from research versions of the technology, such as the larger HiSeq platform, associates Illumina with greater accuracy of base calls and lower indel detection errors (29).

PACIFIC BIOSCIENCES

To compete in the clinical and Dx space, PacBio introduced the desktop RS machine in 2011. PacBio utilizes single molecule real time (SMRT) sequencing. DNA template bound to DNA polymerase molecules is attached to the bottom of 50 nm-width wells termed zero-mode waveguides (ZMWs). Each polymerase molecule carries out second strand DNA synthesis using γ-phosphate fluorescently labeled nucleotides present in the reaction mix. The ZMW width does not allow light to propagate, but energy penetration excites the nucleotide fluorophores in the vicinity of the polymerase at the bottom of the well. As DNA synthesis occurs, the incorporation of each base creates a distinctive pulse of fluorescence, which is detected and recorded in real time (33). In a platform comparison of the three technologies, Quail et al. noted the high fidelity of PacBio data and the ability to read long sequences (28), but added the caveat that very high read depth is required for achieving accuracy near that of MiSeq and PGM. Additionally, in the context of formalin fixed paraffin embedded (FFPE) and fragmented DNA material, PacBio's long read strength may not be of great advantage.

It must be noted that the rapid pace of performance improvement of both the Illumina and Life Technologies benchtop sequencers has been instrumental in making NGS-based Dxs within reach (34). Both platforms have incrementally increased the quantity and quality of base calling while reducing library preparation time and allowing on-instrument primary and secondary data analysis, which was considered the largest bottleneck to clinical and Dx NGS up to early 2011. For example, advances in library preparation have reduced processing times two-fold compared to older version kits available from both companies in 2011. On the instrumentation side, the new, smaller instruments (MiSeq and PGM), have enhanced output and accuracy of base calling compared to the earlier larger throughput NGS instruments (Illumina GAIIx, Illumina HiSeq 2000, and earlier versions of PGM) (28). An Ion Torrent 318 chip with 400 bp sequencing reads can easily produce >1 Gbp aligned data passing Q20 scores. Furthermore, the newer versions of chemistry have significantly improved the average error rates over the length of reads. Also, the design of the new emulsion PCR (ePCR) Ion One Touch 2 system released in late 2012 increased the uniformity of sequencing by enhancing inclusion of low length template Ion Sphere particles (ISPs) in the template and enhancing library templating for sequencing. Additionally, on-instrument analysis improvements significantly reduced the challenges and time constraints imposed by bioinformatic analysis. Although even more improvements are anticipated, these technical advances have made clinical NGS a reality.

OPERATIONAL CHALLENGES FOR NGS ASSAYS IN THE CLINIC

SPECIMEN TYPE AND AMOUNT

One of the key considerations with current clinical NGS tests with Dx aspirations is the reliance on FFPE material. DNA isolated from FFPE specimens presents unique challenges in being highly degraded and of poor quality compared to that from fresh frozen specimens (35). This places a limitation on the size of amplicons that can be reliably amplified from this material, with tests targeting amplicon targeted regions from around 120–180 bp (Ion Torrent AmpliSeq Cancer Hot Spot panel)[1] to ~175 bp (Illumina TruSeq and TruSight assays)[2]. Additionally, DNA derived from FFPE material undergoes cytosine deamination during the fixation process, which can complicate analyses in downstream Dx applications unless a downstream bioinformatic solution is able to address and compensate for such base alterations (36, 37). What is perhaps an equally great challenge is the amount of specimen required for the assay. Ion Torrent assays for cancer mutational hot spot panels

[1] www.iontorrent.com
[2] www.illumina.com

require about 10 ng input of FFPE DNA, the Illumina TruSight clinical assay panel requires 30–300 ng input DNA (as determined by quantitative polymerase chain reaction (qPCR)-based functional DNA assessment) and a majority of the established clinical NGS panels available as lab-developed tests require about 40 μm FFPE material or >100 ng input DNA, in addition to sections for pathology review and tumor markup. In contrast, individual Dx tests using either traditional Sanger sequencing or other PCR-based assays typically require at least 15 μm input per assay. This apparent drawback of large input NGS-based testing (particularly for Illumina assays) has led to methods to reduce sample requirements, such as Rubicon Genomics ThruPLEX kit, Illumina's Epistem technology, NuGen amplification products, and New England Biolabs NEBNext Ultra for low input NGS. Importantly, the assay manufacturers have themselves adopted steps to further decrease input amount for assays without compromising on test sensitivity. One final note: for NGS-based tests, the sample requirement for material is relatively independent of the number of genes in the assay since the test requires the input of a minimal number of amplifiable genomes only (38).

ASSAY TURN-AROUND TIME

A major hurdle in the adoption of a NGS-based test as a CTA is the logistics in terms of the length of time from sample collection to reporting of results. Most clinically applicable NGS-based tests require 7–14 business days TAT (39). In the case of hematological malignancies, such a long reporting time seems to be clinically untenable. Some clinicians are hesitant to use NGS tests for patient stratification and prospective enrollment in trials because patients may not be willing or able to wait 2 weeks for a test result, and thus will pursue other clinical trials in the meantime. As the NGS assay TAT continues to improve (discussed under analytical challenges) this is likely to be a smaller concern in the next few years.

AVAILABILITY OF CROs WITH CLIA NGS CAPABILITIES

Clinical trial sponsors typically prefer to perform clinical trial sample analysis in a single central lab to avoid potential liabilities of using multiple local hospital laboratories, which can compromise results or complicate interpretation due to the use of different tests, different instruments, different validation standards, and quality control (QC) processes, and different histopathological practices such as macrodissection (14, 40). Unfortunately, despite the potential commercial opportunity that available NGS-based multi-gene panels represent, only a few contract research organizations (CROs) or specialty testing labs have invested the effort to develop the expertise to offer NGS services as Clinical Lab Improvement Amendment (CLIA) laboratory tests suitable as CTAs. Thus, the majority of the technical expertise does not reside in traditional central labs and CROs (11), but rather in academic institutions and in large clinical hospitals, where medical practitioners have begun to use NGS-based mutational profiling screening to match their patients to the appropriate therapeutic (41). These factors represent a significant challenge for pharmaceutical companies interested in developing NGS-based CDxs.

The concern about using local laboratory for enrollment to clinical trials comes from several different areas. First, there may be variability due to different interpretation of the various guidelines, checklists, and recommendations available for NGS assays (42–44) since laboratory directors have some discretion and may interpret the rules differently in some cases. An example is the interpretation of the College of American Pathologists (CAP) NGS checklist that recommends orthogonal analytical confirmation of all encountered mutations from an assay before the mutation is reported as clinically actionable (43). This guidance seems to be interpreted differently in different labs based on the availability of subjects, which limits the probability of encountering samples with said mutations. Second, the current lack of standardization between hospital laboratories, especially in analytical and post-analytical processes, introduces risk in, for example, mutation calls for the same samples since they may utilize different platforms, assays, software, and algorithms to make mutation calls. This is even seen for simpler, non-NGS-based assays such as for *KRAS* mutation detection assays. In a retrospective study (29) in which specimens from colorectal cancer patients treated with panitumumab (an anti-epidermal growth factor receptor gene (EGFR) monoclonal antibody) were analyzed for the presence of activating *KRAS* mutations in both local hospital labs and a centralized testing facility at a CRO, the authors found that 6 of the 60 patients tested had mutations and should have been excluded from the study. The conclusion was that the LDTs in local hospital labs failed to detect the *KRAS* mutations, allowing ineligible patients to be enrolled, and thereby diluted the drug response rate since patients with *KRAS* mutations were not expected to respond to panitumumab treatment (45). That this can happen with a simple PCR-based mutation test illustrates the risk associated with complex assays such as NGS-based assays (43).

The challenge for the pharmaceutical company is how to run a clinical trial that maintains the homogeneity of the trial population in light of the paucity of CROs with CLIA NGS capabilities. Some have suggested to use the local lab test as a CTA for enrollment but confirm the result with a centralized assay or to use the local lab test as a screen to identify patients whose samples should be analyzed by the centralized CTA. Both of these suggestions are problematic. First, analyzing the patient specimen by two assays unnecessarily consumes limiting material. Second, discordant calls are inevitable, especially for assays as complex as NGS-based assays. Determining which of two discordant results is accurate will likely be time-consuming and expensive. Furthermore, the discordant data will likely raise concerns of any regulatory agency reviewing the clinical trial and it may call into question the accuracy of the CTA. Similarly, the idea to use local lab assays to screen patients for subsequent central lab testing will definitely introduce a patient population bias if the study only enrolls biomarker positive patients (12), and it may introduce a bias even if the study has both biomarker positive and negative arms. In general, it seems better to focus on reducing the TAT of sample analysis at the centralized laboratory than to rely on local laboratories for patient eligibility decisions.

A new paradigm in clinical NGS testing is the emergence of companies like Foundation Medicine (FM) and Personal Genome Diagnostics (PGD), which offer NGS-based panel tests as CTAs to support clinical trials as well as directly to physicians. Boston-based FM offers the Foundation One panel that reports on the

mutational status of 285 genes that are found to be commonly mutated in cancers; it has also recently announced a similar panel for hematological malignancies (46). PGD, based out of Baltimore, offers a clinical targeted cancer gene panel cancer select for the detection of genetic alterations in 120 well-characterized cancer and pharmacogenomics genes (47). These companies thus offer an alternative to local laboratory testing for clinical trials. Companies can either use one of these commercial panels as a CTA or can establish a clinical trial protocol that enables recruitment of subjects that have already had the tests performed (13, 48, 49).

FDA-CLEARED INSTRUMENTATION

Although Illumina's MiSeqDx instrument received CE marking in June 2013, the lack of commercially available instrumentation was a major hurdle to CDx development prior to the FDA-clearance of Illumina's MiSeqDx platform as a class II device in November 2013 [510(k) number K123989]. In addition, the FDA also made the device and substantially similar devices exempt from the premarket notification requirements. At the same time, the FDA-cleared Illumina's cystic fibrosis carrier screening assay, an assay that detects all 139 variants in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, as well as an assay for CF diagnosis by sequencing all the medically relevant regions of the CFTR gene assay (Source accessdata.fda.gov and illumina.com). The type of data required for these submissions provides the first documented and public view into the Center for Devices and Radiological Health's (CDRH) specific expectations for verification and validation of NGS-based Dx tests; see below for a section in which this is discussed in detail.

Life Technologies' has recently stated that its Ion Torrent PGM Dx System will be registered as a class II 510(k)-exempt device with the FDA, as opposed to applying for 510(k) clearance as was done for the Illumina MiSeqDx (50). This is apparently prompted by the FDA decision that the MiSeqDx instrument and substantially equivalent devices of that generic type will be classified into class II and be exempt from premarket notification requirements [510(k) K123989]. The Ion Torrent PGM Dx will be building on Life Technologies' expertise with Dx instruments such as the 510(k)-cleared 3500 Dx Genetic Analyzer. The PGM Dx instrument will be an open platform for NGS tests but without specific assays submitted to the FDA. Life Technologies has stated that Dxs manufacturers applying for tests on the PGM Dx will reference the master file as needed to support their submission to the FDA and those assays would be evaluated by the FDA through either the 510(k) or premarket approval (PMA) processes. The Ion Torrent system has one significant difference in that it includes two peripheral accessory instruments, the Ion OneTouch Dx for ePCR-based template preparation and the OneTouch ES Dx for magnetic bead-based ePCR library enrichment.

Pacific Biosciences RS II DNA Sequencing System's regulatory path is currently not clear. However, in a significant move recently, Roche Diagnostics and PacBio entered into an agreement to develop Dx sequencing systems and consumables utilizing PacBio's SMRT technology. Per this agreement Roche will become the exclusive worldwide distributor for PacBio's human IVD products (51).

TECHNICAL AND ANALYTICAL CHALLENGES FOR NGS ASSAYS IN THE CLINIC

DESIGN OF THE NGS ASSAY

The first challenge toward a successful NGS CDx is the assay design. Most current clinical NGS assays rely on a hybrid-capture or PCR amplicon-based approach to provide overlapping, high density coverage across regions of interest (52). When working with FFPE biopsy specimens, the number of amplicons needs to be judiciously optimized to allow efficient coverage of large regions while keeping amplicon size small to enable efficient amplification of formalin-damaged DNA (53). The choice of platform and the degree to which the assay needs to include promoter, 3′ untranslated region (UTR), splice sites, or introns also affects assay design. Currently, most commercially available panels only cover exonic regions. While Ion Torrent's hot spot mutation panels cover shorter fragment amplicons, Illumina's exon coverage-based design tends to favor longer amplicons. While overlapping longer amplicons may increase the fidelity of readout by utilizing multiple overlapping fragments per base, amplicon length must be judiciously balanced to enable FFPE fragmented DNA analysis.

Genomic complexity of the region of interest can impact accuracy and precision of an assay (54), so it is also important to understand and to give due consideration to the same in assay design. Since the genome has been shown to replicate at different times with variable error as a function of time of replication, the analytical parameters including error rate must be calculated accordingly for specific regions based on sequence context (55, 56). Knowing whether the region of interest is a region of lower intrinsic fidelity allows one to improve accuracy by compensating with higher read depth. Similarly, the degree to which samples will be multiplexed must be planned into the design to balance read depth (and thus higher confidence in calls) versus the cost of the assay, since higher read depth leads to lower multiplexing capacity and thus increased per sample assay cost (43, 57, 58). Ensuring that the assay design and bioinformatics analysis take into account the region's characteristics, it should be applicable to individual assay developers building Dx assays on other platforms as well. Finally, it is important to develop models that take into account the expected sample throughput, frequency of testing, the assay TAT, and the degree of batching to forecast the optimal multiplexing strategy. For batching samples there must exist guidelines for standard multiplexing and read depth to ensure equivalence of test results.

QUALITY CONTROL STANDARDIZATION

The lack of industry-wide standardization of critical components of QC also represents a challenge for CDx development. The current NGS technologies have higher error rates and novel error modes compared to traditional sequencing, which results in variability in mutation reporting (59–61). Thus, during test development it is essential to have a strategy to detect and reduce the frequency of false positives and then to establish QC procedures to assess test performance, yet there is no established or generally accepted approach (62, 63). This strategy will likely involve varying bioinformatics parameters of the variant calling software and establishing a method to confirm mutation calls with orthogonal

methods. Investigating false positive calls is crucial during assay development and refinement. While Sanger sequencing is still considered the gold standard, its lower sensitivity of detection [around 17–25%; (64)] limits its use for confirming mutations at the low frequencies that are commonly detected with NGS. Multiple strategies for orthogonal validation are possible, such as using a different assay design on the same NGS platform to evaluate design robustness or employing an orthogonal NGS platform with similar sensitivity to identify any platform-specific artifacts. Orthogonal validation with non-NGS platforms such as Sequenom, COLD-PCR, and pyrosequencing may be a preferable approach and these are also gaining popularity as clinical NGS validation strategies (44, 46, 65). False negative calls are more difficult to detect but the utilization of variant call files (VCFs) that report read depth at every position allows for positive confirmation of a wildtype call and not just the absence of a variant call at that position. Second, standardized procedures for QC, including spike-in sequences are yet to be standardized. Some have proposed that spike-in samples should mimic the region of interest in terms of genomic region tertiary structure, interfering genomic regions competing for similar priming sites and, lastly, for genomic complexity, including but not limited to base distribution, presence of similarly presented homopolymeric regions or the known regions of ambiguity such as GC combinations that have been found to complicate variant analysis in a platform-specific manner (29). Recent forums for NGS standardization (43, 44) have discussed the needs for both artificial sequences, which will allow quality assessment of library preparation and analysis (66), and clinically relevant biological mimics, which can faithfully recapitulate biological variation induced by genome complexity as well as serve as a good benchmark for matrix-associated artifacts, e.g., FFPE matrix artifacts. Without industry-wide recommendations or guidance from regulatory authorities, this aspect of CDx development represents a challenge.

CLINICAL AND DIAGNOSTIC RNASeq ASSAY DESIGN CHALLENGES

The use of RNASeq for transcriptional profiling, gene expression studies, identification of variants, and pathological fusion or splicing events (67) is an area of great interest to the clinical genomics community. Clinical RNASeq brings to the fore the capacity to utilize gene expression signatures for highly informative disease sub-type classification or prognosis signature development, as has been demonstrated by gene expression based Dx tests like Agendia's MammaPrint test (68) or Genomic Health's OncotypeDX tests (69). Clinical RNASeq at the whole transcriptome level offers invaluable insight into a patient's transcriptome and associated gene expression changes informative of pre-disposition to cancer or patient stratification strategies. It is especially pertinent for conditions where alternative splicing and isoform selection can affect response to drugs or can predict selective outcomes in response to therapy. RNASeq analysis can be used to develop a robust molecular sub-type signature for a cancer as is apparent from recent studies utilizing gene expression signatures for prognostic and Dx assays (70, 71). In reality, as with issues facing the whole genome sequencing and whole exome sequencing field, it is more likely that targeted panels rather than whole transcriptome offerings will first show clinical utility.

Some of the issues that hinder the adoption of clinical RNASeq are the quality of the RNA from clinical biopsy materials, extremely complex bioinformatics and statistical analysis as well as design of the experiment and its execution in the clinic. The quality and quantification of RNA is critical for successful library preparation and QC controlled analysis of the sample. Clinical FFPE sample-derived RNA is likely to require pre-processing repairs or methodologies to enable low input amplification or enrichment based library preparation. Sample RNA preparation and RNASeq process reproducibility and accurate quantification will have to be highly validated to avoid issues such as prep based biases in quantification of GC-rich transcripts or small RNA species. It will also be important to assess the impact of factors such as RNA secondary structure, the presence of small RNAs in the sample or interfering substances (72). Any lack of read-out reproducibility in a gene-specific manner will hinder the establishment of fold change cut-offs for clinical decision-making (73). Qualifying adequate depth of coverage is critical because accurate quantification of transcripts in clinical RNAseq is dependent on read depth (74).

The bioinformatics analysis of RNASeq in the clinic is considerably more complex than pipelines for DNASeq. For one thing, normalization of data needs to be highly accurate for the technology to be quantitative for the measurement of relative expression values (75). As algorithms for non-clinical RNASeq are improved and as scientists employ better controlled experiments and statistical strategies (76), some of the issues that have plagued clinical RNASeq bioinformatics may be resolved in the near future. Definition and standardization of clinical databases and annotation pipelines is another critical requirement for clinical RNASeq. Currently, because of variability in gene models in different databases such as AceView and RefSeq as well as frequent changes to the databases, non-clinical RNASeq efforts encounter high variability in definition and annotation of regions. In addition, one of the key features of clinical RNASeq will be the ability to identify specific re-arrangements and spliced isoforms. Considering that detection of fusions and gene re-arrangements have high clinical relevance, it will be necessary to develop both bioinformatics methods and mate pair library construction protocols or similar technology but simpler workflows to detect re-arrangements and gene fusions (77). The design of targeted experiments should enable more hypothesis-free quantification of the staggering complexity of gene fusions and transcript re-arrangements possible as well (78). Without such a highly complex identification and quantification strategy the power of clinical RNASeq cannot be fully realized. Targeted RNASeq approaches, particularly with amplicon-based panels, would need to have highly plexed designs to allow a more discovery oriented capture approach while allowing highly sensitive quantification. Hybrid capture based panels would possibly offer more robust splice isoform coverage but suffer from more labor intensive protocols.

Reference materials, controls, and QC standards need to be defined for clinical grade RNASeq in the same way these are becoming standardized for clinical DNASeq. An advantage for the clinical RNASeq field is the availability of the highly qualified human reference MAQC-A and MAQC-B reference materials and the extensive data on tissue-specific expression of potential housekeeping genes from exhaustive microarray profiling (79).

This approach has been utilized to test and aid data correction in RNASeq in research settings and may find easy integration into clinical practice as well (80). Recently, the set of eukaryotic mRNA mimic Spike-In Control Mixes developed by the External RNA Control Consortium (ERCC) has been suggested as a clinically useful control option. These have pre-formulated quantified blends of 92 transcripts derived from National Institute of Standards and Technology (NIST)-certified DNA plasmids. The call for a MAQC-like platform comparison for RNASeq to identify issues and to evaluate platform-specific biases or strengths is being addressed by at least two consortia, the FDA's SEQC (MAQC-III) group and the Association of Biomolecular Resource Facilities – Next-Generation Sequencing (ABRF–NGS) group study. These results will be highly informative to the developers of clinical RNA sequencing (RNA-Seq) assays.

An emerging theme in the translational NGS community has been the utilization of RNASeq for detection of mutations (81, 82). Analysis pipelines that can account for factors like editing biases are not publicly available or are not sufficiently validated to allow such analysis in a clinical context, but once achieved these may offer a highly efficient method for capturing both mutational and expression level information in the same analysis (24, 83). Increasingly, studies that compare the benefits of both types of studies in combination with even epigenetic and microRNA signatures of the tumor for comprehensive profiling are likely to gain traction. The use of RNAseq instead of clinical DNASeq is likely to require a significant effort that includes matched RNAseq–DNAseq analysis and the development of sophisticated algorithms for analysis. Nonetheless, it appears likely that for at least certain molecular sub-types RNASeq-based gene expression profiling and analysis may provide a more predictive result than mutation based analysis alone.

POST-ANALYTICAL CHALLENGES

BIOINFORMATIC MUTATION CALLING ALGORITHMS

One of the major hurdles to adoption of NGS for CDxs is the current state of variability in the performance of variant calling software depending upon the bioinformatics pipeline used (84, 85). It is a routine occurrence that variations in mutation detection are observed from the same raw data set when utilizing different algorithms for variant calling, even with the assumption that similar pre-variant calling processing was performed on the final dataset (86). **Figure 1** is a high level schematic illustrating the basic steps in a bioinformatics pipeline to stress the number of steps and the complexity of variables that impact mutation detection. The initial sequencing data (DAT files) are derived from Illumina imaging data or Ion Torrent pH change related voltage data. Basecall (BCL) files contain data where the sequencing data (images or voltage) have been translated into a nucleotide call. Multiplexed data are then separated into per sample data via the sequencing index identity and FASTQ files are generated, which contain sequencing read data that include the sequence and an associated per base quality score, called a phred score or Q score (87, 88). Reads are then aligned to a known reference sequence containing genomic coordinates and organized into BAM files (89). Variation analysis, or variant calling, refers to the assignment of

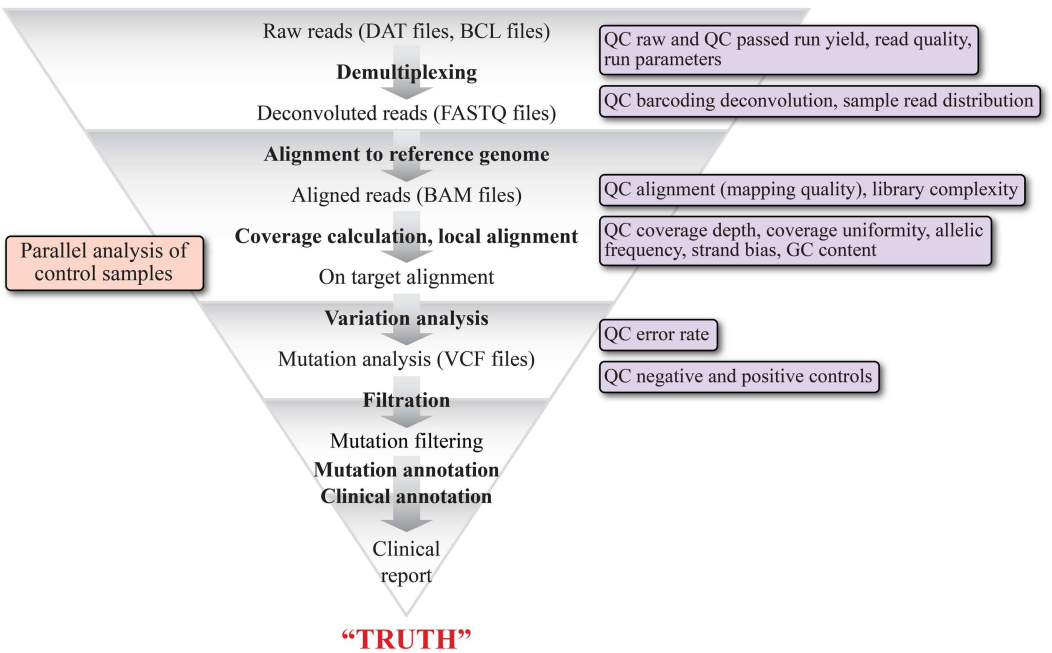

FIGURE 1 | Schematic representation of the various bioinformatics and statistical analysis steps of a typical clinical NGS variant detection data pipeline. The graphic illustrates the major modules of the pipeline and their output file types, beginning with raw reads (DAT files) and ending with a clinical report. The pipeline is highly tunable, as each of the steps can be optimized by adjusting parameters specific to each step. The triangular shape is intended to convey that each step acts as a filter to remove reads that do not represent variants. The key quality filters that can be applied are shown in the boxes to the right.

non-reference status (i.e., a mutation or a variant) to a specific queried position in the genome and generates a tab separated VCF. The variant calls are filtered to minimize false positives and false negatives while maintaining the sensitivity and specificity of the data by utilizing the phred quality scores, which vary on different platforms (63). To generate a clinically actionable report, the high confidence variants are unambiguously annotated based on clinical data showing a causal relationship between the variant and disease and with information about the variant in the literature (90, 91). A vast variety of software is available for each step of NGS data analysis, as are a number of bioinformatics suites designed specifically for Dx testing and which can be tailored to provide a streamlined, module locked analysis for Dx processing (63, 91). Some suites may also allow the user to change settings for test development purposes. Recently, the NIST spearheaded an effort (92) to develop a highly confident variant caller by encouraging the NGS community to share sequencing data of their NGS reference material NA12878 (v.2.15). This effort should greatly aid the standardization of analysis methodology and better QC for assessing false positives and false negatives (66, 92).

The traditional regulatory framework makes integration of the NGS data analysis software into the Dx device system imperative, with a fixed version of the analysis algorithm for the regulatory submission. This presents a challenge for the device developers since variant calling software applications are continually evolving, particularly in the ability to detect indels, in efforts to reduce analysis time and in the use of control set parallel analysis (41, 85, 86, 93). As new versions of variant calling software with better sensitivity and specificity become available, it is reasonable to assume, based on current precedent, that new 510(k) submissions will be required for these devices.

Standardization of data QC and filtering, variant detection and annotation of samples is imperative for developing Dx tests. Ideally, NGS-based data analysis should be subjected to rigorous internal and external QC with rules to accept or reject data akin to Westgard rules (94, 95) used for other analytical tests. The field is still open for discussions on how these rules should be implemented for NGS-based CTAs and Dx tests. For example, are traditional Westgard rules applicable to a quantitative parameter of NGS-based mutation detection tests such as mutation allele frequency? If not, then what type of quantitative rules can be used to establish in control processes? It is imperative for the field to define the type of control samples and the QC procedures to accept or reject runs. Some laboratories argue that internal control targets must also be met prior to a decision to report mutations (43, 85).

Another novel aspect of NGS mutation calling is that variants are rated based on the certainty of the call (87, 88). Phred quality values are assigned to specific steps in the process such as base calling and read alignment. Read depth, read quality, frequency of detection of the allele, strand bias, annotation as germline variant or variant of unknown significance, or lack of "actionability" all can be used to assign a confidence score to a particular call (57, 89, 96). Segregation of variants per characteristics of read depth, base quality, read quality, and strand bias are easily automatable with most Dx instruments available, but current software programs do not provide easy readout of mis-alignment-based read drops, reads that are exempted from final analysis by homopolymer-based inaccuracies, reference allele bias, or reference genome bias (60, 61, 97). These are post-analysis computing requirements that still need to be built into software to minimize operator involvement.

It is interesting to note that each sequencing platform has its particular advantages and drawbacks in terms of regional biases that complicate variant calling. In the past, Illumina MiSeq data have been associated with high accuracy but increased strand bias with GC-rich motifs, as well as low accuracy for homopolymer stretches beyond 20 bp (97, 98). In the November 2013 FDA 510(k) Decision Summary for the MiSeqDx instrument (Number k123989), Illumina specifically claims the ability to detect single nucleotide variants (SNVs) as well as deletions up to three bases. Based on a very limited data set, the instrument can also detect 1 bp insertions, but this is limited to non-homopolymer regions, since the MiSeqDx instrument was shown to have problems detecting 1 bp indels in homopolymer tracts, e.g., polyAs. The notification also states that Illumina's current MiSeqDx analysis software will automatically remove any homopolymer tracts of longer than eight continuous identical bases (R8 error). Interestingly, the MiSeqDx instrument claims to be a qualitative detection platform rather than quantitative. The MiSeq has generally been reported to have higher fidelity for indel calling than Ion Torrent (28, 61, 99). Ion Torrent homopolymer regions beyond 20 bp tend to be misaligned and discarded so that alignment algorithms must be optimized per region of interest to allow inclusion of misaligned regions (32, 61). The Ion Torrent Dx platform specifications will become clear when it is registered. Strand bias related inaccuracies and decreased depth of coverage or uneven coverage (due to allele dropout in case of sampling error or as a function of tumor heterogeneity) can also compound the problem of mutation calling inaccuracies. Accurate base calling algorithms for Dx assays must minimally utilize spike-in controls during technical feasibility experiments and raw data controls for software training that include mutation calls in regions of predicted poor base calling if those are part of the assay design (41, 43, 66). The use of a highly sequenced reference sample, such as NA12878 by NIST (v.2.15) for software training and algorithm development has been proposed in many forums such as the NIST "Genome in a Bottle" Consortium (92). Recently, the same was used by Illumina to demonstrate accuracy in its MiSeqDx platform 510(k) submission application. Additionally, it is reasonable to propose to include engineered mutations as part of spike-ins where inaccurate calls may result due to biases from GC-rich motifs, strand bias, reference allele bias, homopolymers, and regions of low coverage if down-sampling total calls for normalization, etc. For assessing the accuracy of the data pipeline, normal/reference sample pairs may be developed as proficiency testing (PT) material. Alternatively, specially designed artificial DNA mixtures that contain the majority of expected mutations (from literature and clinical findings) should be used as reference material in accuracy, sensitivity, and precision studies in the technical feasibility phase. The National Cancer Institute (NCI) initiative to make specific mutations available as plasmid constructs as well as the availability of characterized mutant DNA or recombinant tissues from companies like Horizon Dx are allowing test developers to devise such experiments with spike-in-based QC (43, 66). From its recent guidance on Personalized Medicine, the FDA also seems to acknowledge that testing of variant calling

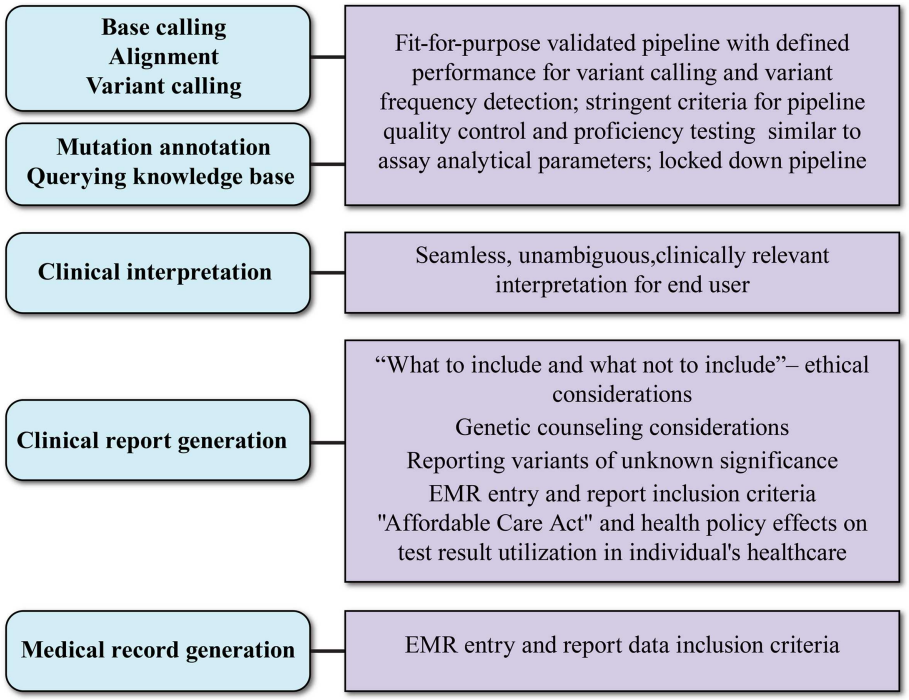

FIGURE 2 | Aspects and key considerations of clinical NGS data reporting. Main aspects of clinical data reporting are shown in ovals to the left; key considerations are shown in boxes to the right. The uppermost three aspects rely on the bioinformatic pipeline. What test results are reported in the clinical report (fourth oval) is influenced by socio-ethical considerations and may require genetic counseling and support systems. The evolving payer landscape and medical records guidance will affect how NGS clinical reports are captured in patient records.

for a specific set of mutations and the establishment of the platform's sensitivity and specificity may be sufficient for the clearance of a NGS-based regulated device. One novel aspect to the application of NGS-based tests is the need for a standardized set of raw data for mutation calling algorithm development. To meet this PT need, the NIST Genome in a Bottle Consortium as well as CAP have both been actively advocating availability of public data sets from extremely well studied samples as PT material to assess a particular pipeline's sensitivity and specificity in mutation detection to avoid lab to lab variation in mutation detection.

In addition to bioinformatics analysis for variant calling, there are several aspects of data interpretation and annotation that must be standardized for NGS tests to be adopted into clinical practice. These are graphically represented in **Figure 2** and are discussed below.

DATA REPORTING

If the FDA requirement for a NGS-based Dx approval is demonstration of accuracy and precision for each assayed base, it is possible that Dx developers may choose to limit the reportable content of a NGS panel by utilizing base masking in an effort to reduce the extent of analytical validation efforts. In the recent 510(k) application for the MiSeqDx instrument and the CFTR gene Dx test on the instrument, data showing the orthogonal validation of a subset of base positions was accepted, suggesting that the FDA may only require a sponsor to show performance data for the unmasked, reportable nucleotide positions on future submissions of panels or single-gene assays. It will be interesting to note the Agency's guidance on this topic since the masked data could potentially still be utilized for analysis to develop or enhance predictive mutation signatures on retrospective analysis.

Another key consideration for data reporting is the reporting of variants of unknown significance. The ACMG guidelines from 2008 (100) defined various cases of variants of unknown significance including: (1) previously unreported variations with possible ramifications for the disease being studied. This includes indels, frameshift mutations, and invariant splice site AG/GT nucleotides variants that can alter the reading frame and thus the expressed gene product. (2) Previously unreported variations that may or may not be causative of the condition. These are exemplified by missense changes, in-frame indels, and splice consensus sequence variants or cryptic splice sites that may affect regulatory processes, e.g., interruption of splicing enhancers or suppressor sites. In these cases, clarification of the clinical significance of variants is required and it may be important to flag them accordingly in a report. (3) Previously unreported variations that are probably not causative of disease, e.g., synonymous mutations that do not alter protein sequence or affect processing or regulatory pathways, or are found in addition to a variant known to be associated with pathologic change (in autosomal dominant disorders). (4) Previously reported sequence variations that are recognized as neutral variants with evidence available that the variation has been consistently observed in a normal population and does not associate with disease or predisposition to disease. (5) Sequence

variation not known or expected to be causative of disease, but is found associated with a clinical presentation, e.g., variants that contribute to disease as low-penetrance mutations which alone or in combination may or may not predispose an individual to disease or modify the severity of a clinical presentation in complex disorders. For such a category the institute suggests reporting as not definitive mutations and stating that medical management decisions should not be made on the presence of the variants alone. This last is probably the most efficacious solution for reporting NGS-based variants of unknown significance since it allows capturing of the profile without unduly triggering medical actionability. Unfortunately, the current forms of patient consent are usually quite limiting and restrict public sharing and analysis of data utilizing big data analytics. There is clearly a need for patient consent agreements to allow meta-analysis, but this is the topic of the next section, data privacy in the age of big data analytics.

Reporting of incidental or serendipitous findings is another area of complexity for NGS-based tests. Some are proponents of the idea that incidental findings should not be reported at all in clinical sequencing without strong evidence of benefit, while others advocate that any and all variations in disease-associated genes are potentially medically useful and therefore should be reported (2, 17, 41, 44, 46). Recognizing the difficulties of reporting such secondary findings which are medically important but unrelated to the reason for test ordering, the ACMG constituted a special Working Group on Incidental Findings in Clinical Exome and Genome Sequencing to make recommendations for addressing such findings in pretest patient discussions, clinical testing, and the reporting of results (101). In the case of targeted oncology panels, this may not be an issue unless specific loci are associated with enhanced risk for other conditions or where particular polymorphisms can affect existing health care routines and drug regimens. Currently, the ACMG working group has only recommended reporting those incidental findings for which preventive measures or treatments are already available or for disorders in which patients are asymptomatic despite the presence of pathogenic mutations. Generally, the recommendation was to report pathogenic variants as incidental findings, e.g., those where the "sequence variation is previously reported and is a recognized cause of the disorder" or "sequence variation is previously unreported and is of the type, which is expected to cause the disorder" (100). These two were chosen no doubt because the group recognized that attempting to report and interpret variants of unknown significance as incidental findings would be particularly challenging. The report also stressed that identification of monogenic diseases via a clinical NGS panel as an incidental finding is highly improbable by current practice.

PRIVACY OF AND ACCESS TO PATIENT RESULTS

Ever since the report that individuals could be identified from anonymous NGS data (102), privacy groups have been justified in their concerns about having sensitive data made public as a result of inappropriately controlled data and reports. Privacy of patient results is also linked to maintaining the highest standards for patient consent to NGS-based testing, anonymized data generation, secure data storage, encryption, and transfer processes that meet the highest standards data (103). The converse of this concern relates to the data that reported back to the patient, especially incidental findings unrelated to reason for which the test was performed. In contrast to whole genome sequencing, oncology-based panels are focused on tumor specific genes assessed in the context of the tumor. They have less content with associated incidental findings and thus are less likely to trigger traditional socio-ethical impact (104). However, an issue which lacks resolution is the reporting of low frequency mutations for which the allele frequency based drug action has not been studied. For example, the technical sensitivity of an assay may allow the detection of a mutant at 0.1%, but there is no framework with which to interpret such a finding, and reporting it to the patient may cause more harm than good.

INTERPRETATION OF RESULTS

The mainstream adoption of NGS Dxs will rely heavily on easily interpretable test results. One critical aspect of data interpretation with NGS-based tests is the comparative reference human genome. This is an individual genome and may not be an ideal reference genome for most individuals in the population. For this reason, some commercial NGS providers have started stressing the need for a matched germline control comparator sample such as peripheral blood or normal adjacent tumor tissue from tested individuals. The constant evolution and enhanced annotation of the reference genome as sequencing-based studies continue to reveal new genomic complexities also confounds interpretation. In the example from the MiSeqDx 510(k) decision summary, it is interesting to note that a compound reference genome derived from two well-characterized samples was utilized in addition to human genome build 19 [NCBI Human reference February, 2009 (GRCh37/hg19) assembly] [FDA 510(k) K123989 decision summary]. For example, the two genomes differed in a particular homopolymer run, which was a run of 14 A's according human genome 19, while the sequence in the composite reference genome had a run of 15 A's. This was significant because it directly impacted interpretation of the MiSeqDx sequencing accuracy study, since all 13 samples analyzed were reported as having 1 bp insertions since 15 A's were detected in all 13 samples. As new variants and polymorphisms are identified, it may be warranted to re-annotated or re-issued reports to include the new data or its new interpretation.

OVERVIEW OF DIAGNOSTIC TEST REGULATORY APPROVAL PROCESS

As a prelude to the regulatory challenges, we digress to provide an overview of the Dx test regulatory approval process. The basic regulatory pathway options for Dx device development are summarized in **Figure 3**. This section describes a generic IVD submission process with the authors' comments on possible paths for NGS-based devices.

For any given test that is submitted for FDA consideration, the route to commercialization may be via a 510(k)/pre-market notification process or via a PMA application. The decision to take a NGS-based clinical test via the 510(k) or PMA process will depend largely upon the perceived risk associated with the Dx device. The 510(k) Dx IVD process relies on the presence of a predicate device or devices. However, FDA has utilized the *de novo* 510(k) pathway when the risks of the new device are consistent with other

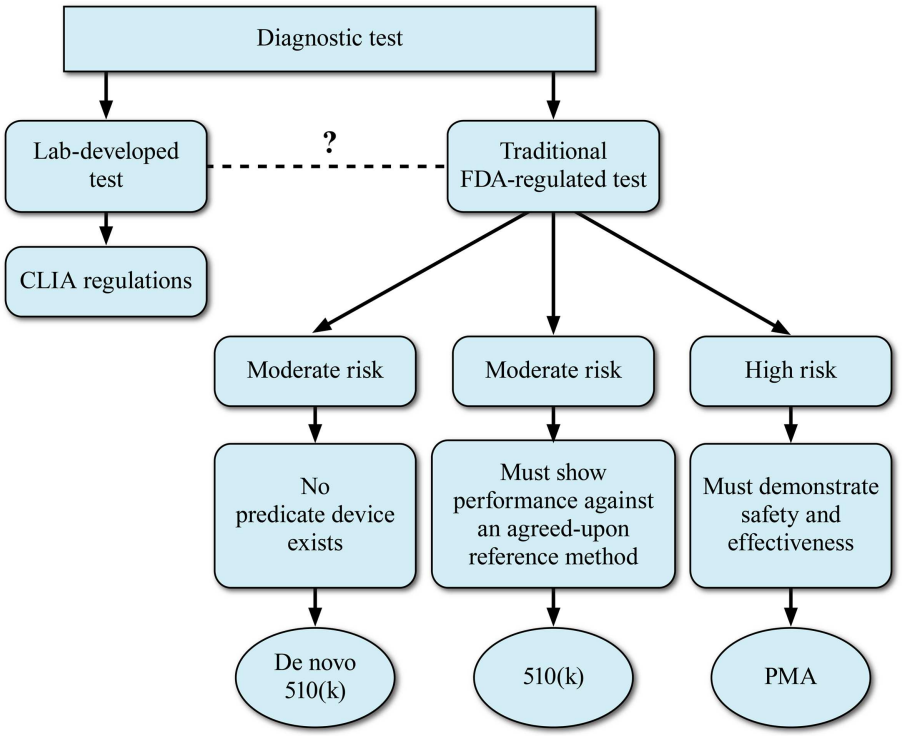

FIGURE 3 | Regulatory models for development of NGS-based diagnostics. The FDA device classification for a regulated NGS-based diagnostic device will depend on the perceived risk associated with the diagnostic device.

510(k)-cleared devices but a clear predicate is not available. The 510(k) process may be appropriate for those NGS-based tests that will be utilized for monitoring disease or for tests where the perceived risks are lower. Although the concept of a predicate device is woven into FDA's device regulation, the reality for the genetic tests that have been cleared or approved to date is the new system is not compared head-to-head with a previously cleared system. Rather, the new method is compared to a gold standard method, which is considered truth. For most DNA applications, the gold standard has been bi-directional Sanger sequencing. Applications which have relatively higher perceived risk to the patient, such as NGS-based oncology tests, will likely be required to go the PMA route to demonstrate safety and efficacy. In these cases, a reference method will also be used to demonstrate accuracy of the device.

A PMA submission for a CDx NGS test will entail coordinated review of the drug by the Center for Drug Evaluation and Research (CDER) and of the device by the CDRH (or CBER for certain disease indications). The IVD developer will have to demonstrate the safety and effectiveness of the *in vitro* Dx device when used as specified in the label. The Dx device must be considered as an entire Dx system including reagents, hardware, software, data analysis, and result reporting. Use of the device in the pharmaceutical clinical trial will provide important data to demonstrate clinical validation of the assay. Although NGS IVD submitters may have to undergo an advisory panel review regarding clinical, regulatory, scientific and statistical issues due to the novelty of the NGS platform and assay structure and readout, it seems doubtful since other CDx applications have not had this hurdle and FDA has seen fit to clear the Illumina platform with no such advisory panel requirement. For an approved PMA any modifications to the test or device, manufacturing process, its labeling, intended use or sensitivity or specificity would require FDA notification and prior approval. In general, it is imperative that NGS-based Dx stakeholders seek clarity utilizing pre-submission meetings with the CDRH, and specifically the Office of *In vitro* Diagnostics and Radiological Health (OIR), well in advance of trial planning. It is important to engage in such discussions early as FDA thinking is evolving rapidly.

Many of the regulatory challenges for CDxs are not unique to NGS. Although NGS tests may be more complex than other technologies, the same principles will apply. The FDA's expectations on the analytical validation and performance characteristics of NGS-based assays will differ somewhat for each individual assay. However, the 510(k) clearance of Illumina MiSeqDx reveals some aspects of the regulatory agency's viewpoint on validation. Since this is the crux of the regulatory challenge, we summarize in detail the main aspects of Illumina's 510(k) submission studies [510(k) summary, e.g., K124006, November 2013] as early pointers to the type of experiments FDA may expect.

510(k) CLEARANCE OF ILLUMINA MiSeqDx

With the MiSeqDx clearance, the FDA has given some indication the type of information that will be required for approval a NGS-based CDx for tumor mutation status. First, the 510(k)

summary indicates that accuracy data for all claimed specimen types and nucleic acid types were required. Two sources of well-characterized samples (based on well validated sequencing methods) were queried with all of the claimed sequence variation types, types of sequencing and with the sequences located in varying sequence context (e.g., different chromosomes, GC-rich regions). The 510(k) summary indicates that sequence data generated with a sequencing technology platform and variant calling method independent of the device manufacturer is required for at least one of the reference samples. Percent agreement and percent disagreement with the reference sequences were described for all the regions that were queried by the instrument. Illumina performed accuracy testing in three studies. The first assessed overall accuracy over a wide portion of the genome by utilizing 13 very well-characterized samples from parent–child triads that had been sequenced by multiple laboratories and multiple sequencing technologies. Human reference genome 19 was used to assess accuracy across 24,434 bases on 19 chromosomes encompassing a variety of genes containing potentially clinically relevant exons. The second study assessed the accuracy of the MiSeqDx instrument at 17 highly confident variant calls in the NIST NA12878 standard reference material. The third accuracy study assessed the instrument's performance in detecting small insertions and deletions by analyzing six samples using the Cystic Fibrosis 139 Variant Assay, which included a subset of clinically significant indels in *CFTR*. The detected insertions and deletions were all confirmed with bidirectional Sanger sequencing as the reference method. Such accuracy studies helped Illumina define its performance specifications for homopolymer stretches, nucleotide repeat regions, and ability to detect indels.

For precision/reproducibility studies, the 510(k) summary indicates that data should be generated using on multiple instruments, with multiple operators and at multiple sites, and that performance data are required for all claimed specimen types, nucleic acid types, sequence variation types, and types of sequencing. As discussed in the Assay Design, a special emphasis was given to variants located in varying sequence context, such as different chromosomes and GC-rich regions, along with a requirement to utilize a high confidence reference sequence data. To this end, Illumina performed three precision studies. For the first study, 13 well-characterized sequenced samples were analyzed in 9 runs using 3 different MiSeqDx instruments and 3 different operators. Samples NA12877 and NA12878 were run in duplicate to assess repeatability. Ninety-four samples and two non-template controls were tested across three lots to establish lot-to-lot reproducibility of the Illumina universal reagents. Each lot was split into two 48-sample runs to test reagents and all possible index primer combinations. All sequencing runs were completed by a single operator and on a single MiSeqDx instrument to remove potential variance contribution from operator or instrument. The MiSeqDx Cystic Fibrosis 139 Variant Assay reproducibility study involved a blinded study with three trial sites and two operators per site. Two well-characterized panels of 46 samples each were used for testing. These contained a mix of genomic DNA (gDNA) from cell lines with known variants in the *CFTR* gene and variant containing cell lines spiked into leukocyte-depleted blood to assess variability from the gDNA extraction steps.

Illumina also addressed the issues of sample cross-contamination (carryover) and intra-run performance. For intra-run performance, a 48-sample library of two samples with unique variants arrayed in a checkerboard of an alternating high concentration (500 ng) and low concentration (100 ng) input was utilized. For inter-run carryover 2 libraries were prepared each with 47 replicates of a single gDNA sample and 1 no template control (NTC). The samples were unique in each library and continuous run assessment was performed to demonstrate absence of carryover. The reproducibility and accuracy of multiplexing was also tested with 12 indices (barcodes) per sample sequenced. Accuracy for all sample/index primer combinations was confirmed as 100% by Sanger bi-directional sequencing and PCR-based confirmation.

For testing the contribution of common interfering substances to variability, four endogenous interfering substances (bilirubin, hemoglobin, cholesterol, and triglycerides) were spiked in eight unique whole blood samples. Blood collection variability and gDNA sample preparation variability were also evaluated, along with sample input amounts, thermal cycler effects, and sample stability. DNA extraction methods were assessed using 168 specimens (14 samples $\times$ 2 operators/extraction method $\times$ 3 runs/operator $\times$ 2 replicates/extracted gDNA sample).

The MiSeqDx approval gives insight into some of the regulatory expectations for NGS-based assays and is summarized here with some general headers for reader clarity:

Specimen and processing-related validation:

(i) The specimen type(s) as source of nucleic acid.
(ii) The type(s) of nucleic acids (e.g., germline DNA, tumor DNA).
(iii) The nucleic acid extraction method(s).

Sequencing variation-related validation:

(i) Type(s) of sequence variations (e.g., SNVs, insertions, and deletions).
(ii) Type(s) of sequencing (e.g., targeted sequencing).
(iii) The read depth required for the sensitivity being claimed and the validation data that supports those claims.
(iv) Accuracy and precision of the test and the types of sequence variations that the test cannot detect with the claimed accuracy and precision (e.g., insertions or deletions larger than a certain size, translocations)
(v) The upper and lower limit of input nucleic acid to achieve the claimed accuracy and reproducibility.

The MiSeqDx instrument's current *de novo* classification is for qualitative assessment for profiling of peripheral whole blood samples, which tend to be of a higher quality. It is important to note new tests, including CDx devices, on the platform are likely to require PMA submissions, especially for tests utilizing heterogeneous samples like tumors. The current MiSeqDx clearance for qualitative results opens the discussion on what further validation strategies may be required to achieve quantitative detection of mutations (e.g., quantitative allele frequency), which may be one of the strengths of clinical NGS.

UNANSWERED ANSWERED REGULATORY QUESTIONS

HOW MANY MUTATIONS WILL HAVE TO BE CLINICALLY VALIDATED?

The FDA has hinted at possible accuracy requirements for complex, multi-analyte specific assays, genes, and panels at the DIA Meeting on Personalized Medicine and CDxs (November 6, 2013). This provides important insight for CDx applications involving tumor suppressor genes and certain oncogenes since actionable mutations may occur anywhere along the length of the gene. FDA has suggested three potential strategies:

- Sequence clinical samples from the intended use population and compare to reference method results.
- Sequence procured samples that span the relevant classes of variants and compare to reference method results.
- Sequence well-characterized reference sample(s) and compare to reference sequence.

CAN AN NGS MULTI-GENE OR MULTI-TRANSCRIPT PANEL BE APPROVED AS A DIAGNOSTIC PLATFORM, ALLOWING MULTIPLE CDx SUBMISSIONS?

At the 2012 Friends of Cancer Meeting the FDA publicly indicated their interest in reviewing NGS-based cancer panels similar to the panels that have been cleared as microbiology devices (i.e., devices that detect multiple viruses and bacteria in a single product) (105) (focr.org). Although the details of this type of submission would need to be worked out between FDA and an individual sponsor, it seems likely that some level of clinical evidence would be needed for each gene or mutation included on the panel. It is possible, similar to the cystic fibrosis assays, that this list could be developed based on medical input and literature. From that point, more specific claims about individual genes could be made on a gene-by-gene basis including CDx claims if the product has been used as part of a clinical trial investigating a particular drug. It is likely that any cancer panel would be subject to a PMA [rather than a 510(k)], and amendments to the original PMA with additional claims on a per panel member basis would be a rational approach to updating the claims for each new CDx.

HOW WILL EXISTING GENOMIC DIAGNOSTICS ALIGN WITH APPROVED NGS-BASED DIAGNOSTICS?

Currently, the intended use statement for each of the Dxs that have been approved in conjunction with a drug list the drug name in the intended use statement. It is reasonable to expect that this policy will continue and that in order for a drug and Dx to be co-marketed the drug and device will need to be linked. Even if there are multiple devices available for testing in conjunction with a specific drug, any of the approved devices will be allowed.

WHAT IS THE APPROPRIATE ORTHOGONAL TECHNOLOGY?

What is the true measure of truth when comparing discordant results? FDA has shown with the recent Illumina clearance that they expect NGS-based mutation calls to be confirmed by an orthogonal technology (in many cases bi-directional sequencing). However, disagreement exists within the NGS community as to what is true orthogonal validation of a NGS-based mutation call (17, 64, 106). The enhanced sensitivity of mutation detection down to 1–5% allele frequency implies that orthogonal validation will require a platform with similar sensitivity. While Sanger sequencing is being used to support mutation validation, for example in the Illumina MiSeqDx 510(k) clearance, it is not possible to use Sanger data to provide a definitive call when mutations in the range of 1–15%. Generally, if NGS and Sanger give discordant results labs tend to use tie breaker tests such as pyrosequencing or Sequenom-based sequencing on the MassArray system. Both of these technologies can detect mutant allele frequencies down to 5–10% frequency and are finding increasing usage in NGS validation. As Sequenom and pyrosequencing vendors create niche products tailored for NGS validation these will likely integrate into clinical NGS workflows. The FDA has shown flexibility in allowing use of these types of technology as orthogonal methods when Sanger is not sensitive enough. However, the FDA will insist on appropriate validation of these methods and will expect to review these validation packages as part of the review process.

Another approach, likely to be costlier but with the opportunity to have near equivalent sensitivity of detection, is the utilization of a second NGS technology for confirmation of assay results, e.g., utilizing both the Illumina and Ion Torrent platforms where the difference in underlying technology make a confirmation of positive results quite robust. The main issue to be cognizant of is the need to adjust analysis parameters to provide equivalent performance with respect to mutation call sensitivity, since each platform uses its unique quality score for data quality assessment. For example, while Ion Torrent recommends using a phred value of Q20 (99% specificity) for high confidence variant analysis, Illumina recommends at phred value of Q30 (99.9% specificity) for ensuring high confidence calls (61). The difference in acceptable phred scale values arises from differences in platform technology, related background signal and noise calculation algorithms (107).

STRATEGIC CHALLENGES FOR DRUG AND DIAGNOSTIC DEVELOPERS

Developing any CDx can be enormously challenging, as seen in the development of the *BRAF* mutation (108) and *ALK* gene fusion (109, 110) tests. A primary reason is that the device development timeline does not align with the drug development timeline, as illustrated in a development timeline chart (**Figure 4**) (15, 108, 111). Ideally, CDx development for the NGS assay would start with the initiation of early phase studies (Ph1/2a studies in **Figure 4**) to allow sufficient time for development of the Investigational Use Only (IUO) version of the device before start of the phase 3 pivotal trials. But this is not often the case, and compromises and work-around strategies are sometimes necessary. Thus, in another example of navigating the rapids, pharmaceutical and Dx companies face some unique challenges in NGS-based CDx development, which are summarized in the next sections.

DRUG DEVELOPMENT CHALLENGE 1 – IS A COMPANION DIAGNOSTIC NEEDED?

The first challenge is whether a co-Dx test is in fact required and how a multiplexed RNA- or DNA-based NGS panel would fit into the traditional CDxs scheme. While a CDx may uniquely position the drug in the marketplace, the overarching reason for developing

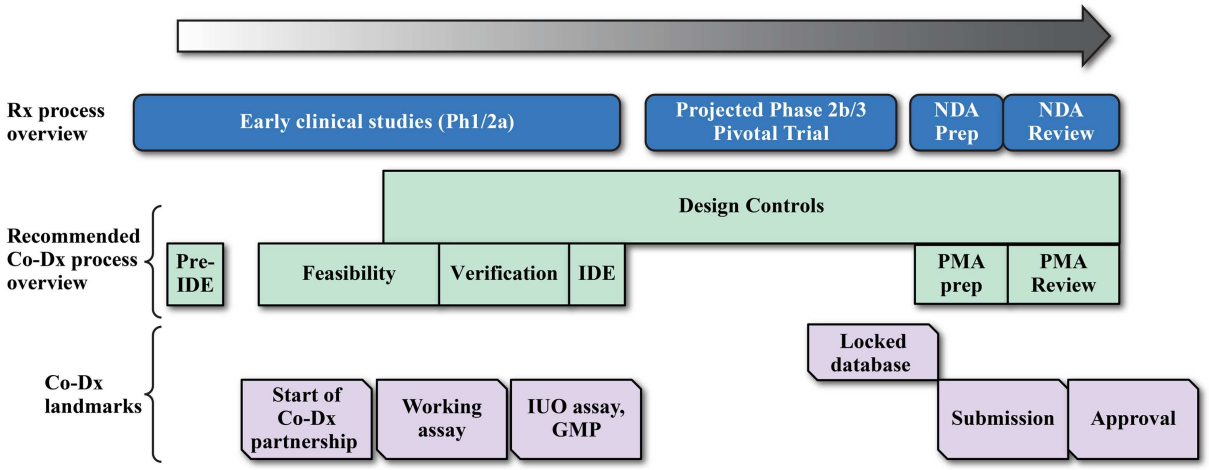

FIGURE 4 | Coordination of drug and device development for a successful companion diagnostic submission. Drug companies and diagnostic developers may work together in several different cost sharing and assay development landmark payment formats for the development of the final IVD product.

a CDx is because it is required for the drug approval. The current FDA guidance dictates that if the test is necessary for the safe and effective use of the drug, then a co-Dx is required. The key factor to determining whether a CDx is required is the efficacy of the drug in a biomarker negative population. If efficacy in the biomarker negative population is sufficient for drug approval, then a CDx may not be required, at least for an initial approval. Thus, this question should be answered early in drug development (12, 109). It appears that NGS-based CDxs will be more relevant in the near future in certain oncology indications where genetically targeted therapies are currently prevalent, such as lung cancer, breast cancer, and colorectal cancer.

DRUG DEVELOPMENT CHALLENGE 2 – USE A SINGLE-GENE ASSAY OR MULTI-GENE PANEL?

At one level, the question seems to challenge one of the guiding principles of Dx development: the simpler the better. Analytical validation of a multi-gene assay, as discussion elsewhere in this article, will undeniably be more work than validation of a single gene. Yet, it might be necessary to consider the pursuit of the multi-transcript or multi-gene panel in some cases such as if the predictive biomarker is a set of mutations in the genes on the panel, i.e., if the marker is a signature for response rather than a single Dx gene mutation. The multi-gene panel approach is predicated on two assumptions: (1) that the FDA will permit the sponsor to mask data from genes that are not required for safe and effective use of the companion therapeutic, and (2) that the FDA will permit different levels of rigor in the validation of genes on the panel, based on whether they are necessary for safe and effective use of the companion therapeutic. The authors firmly believe that the multi-gene panel is a step toward the "multi-gene Dx/many drugs" model even though the path there is not obvious. One of the reasons that the change from "one-drug/one-gene Dx" model to the "multi-gene Dx/many drugs" model will be so disruptive is that the test results from a multiplexed panel could actually lead to the use of a competitor's drug. This leads to the next challenge of how best to design clinical studies to best take advantage of all the content on the NGS assay.

DRUG DEVELOPMENT CHALLENGE 3 – OPTIMAL TRIAL DESIGN FOR NGS-BASED DIAGNOSTICS?

By definition precision medicine focuses on a subpopulation of patients expected to respond to a given therapeutic. Sometimes the population can be quite small, as in the case of metastatic lung cancer patients with the ALK gene fusion, for which crizotinib is indicated (112, 113). Only about 5% of lung cancer patients have the ALK gene fusion (113, 114), which means a great deal of screening was required to identify and enroll patients in the crizotinib studies. This was very inefficient compared to a "basket trial" design (115) in which patients are screened simultaneously for a large number of genetic aberrations using a multi-gene panel to determine their eligibility for a large number of clinical trials involving different therapeutic interventions. Some forward-looking models in this area propose a multi-institution collaboration that employs a multi-gene panel assay in which the cost of the screening assay (including validation) is shared by different drug development entities (49, 115, 116). While this approach would significantly reduce the cost of screening patients for rare subpopulations of patients in PhII and PhIII trials for each individual company, it presents the equally interesting question of whether drug developers will collaborate with competitors in such basket trials. The Friends of Cancer Research initiative for enrollment of patients with advanced NSCLC into trials matched by their tumor profile is one of the first examples of this kind of study (49). The trial seeks to utilize a NGS panel-based approach for enrolling patients into the most suitable trial using an adaptive trial design that allocates patients to suitable drugs from different pharmaceutical participants. It includes five drugs from five different companies and will employ the FM NGS-based panel assay to guide subject assignment and is expected to launch in spring 2014 (116). Overall, drug developers and Dx companies will have to work together to navigate this disorderly transition in testing paradigm (12).

DRUG DEVELOPMENT CHALLENGE 4 – WHEN TO COMMIT TO CO-DIAGNOSTIC DEVELOPMENT?

An important question arises as to when the pharmaceutical company should invest in the NGS co-Dx development process. The best guidance would dictate that CDx assay development must begin at least 18–24 months prior to the start of the registrational studies to allow sufficient time for development of the IUO assay for demonstration of clinical utility in the registrational trial. The Dx development plan depends on many factors such as complexity of the assay, cost of pre-investment, strength of the data confirming the biomarker hypothesis, as well as timeline of drug registration (e.g., whether a traditional Phase 2 to Phase 3 transition timeline is expected) (117). Therefore, variation to the ideal development timeline is often observed and drug companies and Dx developers utilize different developmental strategies to develop the final IVD product with significant investment by both parties (**Figure 4**). Development of a CDx test typically links the market uptake and return on investment of Dx device to the performance of the companion drug in pivotal clinical trials. As a consequence, the cost of development may require creative cost sharing and milestone payment agreements between the pharmaceutical and Dx partner. Some of the plausible developmental strategies possible for current NGS-based Dxs may be summarized as follows:

(i) Linear, risk-averse development model: in this model, development proceeds by a linear, logical flow, minimizing investment risk by delaying decisions as long as possible. CDx development is only begun after the need for a CDx is unequivocally established or until after initial data show the therapeutic has efficacy. Although avoiding pre-investment in Dx development until it is clearly needed may appear to be wise, in reality this may be a poor strategy because once it is clear that the drug is effective, there will be a great urgency to initiate the pivotal studies. The second aspect of risk aversion is the desire to avoid a bridging strategy for the Dx, i.e., starting the Ph3 studies with CTA instead of an IVD-ready version of the Dx (i.e., the IUO version of the assay) and then transitioning, i.e., bridging, to the IUO version by re-analyzing all (or nearly all) of the samples on the IUO version of the assay. This transition introduces significant risk into the process, so avoiding bridging is a good plan, but the cost is a significant delay in the start of the pivotal trial.

(ii) Pre-investment model: the Dx partnership is finalized and the IVD assay development starts prior to the initiation of the Phase 2 study, allowing sufficient time for development of the IUO assay to be completed prior to the Phase 3 start. In this case, the Dx development risk is low, but the Dx utility and therapeutic development risks are high. This is because the Dx development starts before the therapeutic is shown to be effective and before the Dx is shown to be required. Thus, the key risks are the uncertainty of biomarker's clinical utility and the therapeutic's clinical efficacy from Phase 2 data. Although the therapeutic sponsor partner may essentially partially fund Dx development as part of the Dx agreement, the therapeutic sponsor does not absorb all the risk. Dx companies have limited resources and have to select partnerships most likely to lead to a successful Dx product launch. In other words, one of the risks felt by the Dx company is opportunity cost if the program is canceled for any reason, including the failure of the therapeutic.

(iii) Bridging strategy + partial pre-investment. In cases where the traditional 18–24-month window for pivotal trial start is not possible, this model may be utilized to allow a pivotal trial start in a timely manner. This is a very expensive strategy with the drug sponsor absorbing most of the risk. IVD assay developments starts with a prototype assay (non-NGS or NGS-based) and bridging studies proceed as soon as an IUO version of NGS-based assay is ready. This strategy suffers from having high sample requirements as well as necessitating sample stability studies.

DRUG DEVELOPMENT CHALLENGE 5 – TYPE OF A CDx DEVICE: LDT OR KIT?

Many of the early leaders in precision medicine, realizing the possible complexity of the traditional PMA regulatory path for CDx kit development, may consider the LDT IVD model for their therapeutic that requires an *in vitro* Dx. This scenario could arise if the drug maker wants to avoid a large upfront investment in a CDx effort and has identified a reliable partner that can develop an acceptable assay, support clinical trials and provide worldwide access to the assay in their laboratory. The LDT route might also be selected if the company only recognizes it needs an IVD late in clinical development (i.e., in PhII) and wants to avoid a bridging strategy. Even though an LDT can receive FDA-clearance through the 510(k) process (118, 119), it seems likely that the FDA would require the LDT to go through the PMA process. Thus, the main advantage of the LDT route would be to avoid investment in a traditional kit and to avoid a delay related to the development of the IUO device. The current debate on stricter regulation of LDTs may play an important role in such decisions (75 FR 34463, 2010). Variability in LDT design and the increase in number of LDTs over 510(k)-cleared Dx devices is a growing concern (14), since it would take enormous efforts to standardize LDTs to achieve universally accepted tests. Standardization and strict regulation of CLIA NGS LDTs may be the practical scenario encountered most in next few years. As the FDA's guidance and recommendations for LDT regulation become clear and start getting enforced, the clinical NGS field will see standardization at many diverse levels, e.g., controls used in assays, reagents/panels, assay QC parameters and rules for accepting or failing data, bioinformatics pipelines and biostatistics modules, interpretation of data, reporting of data, etc. Key considerations must include early adoption of the Dx assay, preferably prior to pivotal studies. As discussed under time line constraints and in **Figure 4**, not many current NGS-based assays are suitable as Dxs or are ready to be developed into a regulated Dx.

DRUG DEVELOPMENT CHALLENGE 6 – HOW SHOULD CLINICAL ACTIONABILITY BE DEFINED?

While detection of low frequency mutations is one of the great promises of a NGS-based Dx, detection of very low frequency mutations in a Dx test requires several serious design considerations as well. For example, even if a test is technically able to detect a very low frequency mutation (e.g., $<1\%$), the presence of the mutation may not correlate with therapeutic response

since the majority of the tumor (>99%) ultimately does not carry the said mutation. In this case, reporting of the detected mutation may require special consideration. For example, if the said mutation were present at 5% allele frequency, the Dx might report the mutation present and qualify the patient for treatment with the paired pharmaceutical, but if at 0.5%, it might not. In other words, a scenario is possible where patients with a low frequency mutation detected by a Dx test may be ineligible for a clinical trial due to mutation frequency actionability thresholds (41, 120). However, while not "pharmacologically" actionable, the 0.5% mutation detected would likely require reporting for follow-up. Ultimately clinical utility of low frequency mutations will be demonstrated by clinical response, which will provide clarity on what level of sensitivity of mutation detection is acceptable for drug labeling. Similarly, tumor heterogeneity may reveal mutations in a gene or transcriptional changes that are not yet clinically actionable.

DRUG DEVELOPMENT CHALLENGE 7 – WHAT IS THE EX-US REGULATORY ENVIRONMENT FOR NGS DIAGNOSTICS?

In some situations, the Dx that supports approval of a drug outside of the US will be different than the assay that is approved by FDA. This can be due to a number of factors including the US testing being a lab-based assay or the readiness of the Dx company to support distribution worldwide. Additionally, it is particularly important that the policies governing genetic data collection, reporting, and analysis be clear from the start of a Dx program in a territory. In the EU for example, a CDx is not specifically formally classified, though the regulations may change soon (121). However, the test must be CE marked under the EU IVDs Directive (122, 123). The clinical trial use of the test can then be included in the label following a European Commission approval.

DIAGNOSTIC DEVELOPMENT CHALLENGE 1 – ADDITIONAL REGULATORY GUIDANCE

Through the MiSeqDx decision summary, Dx companies are just now getting a glimpse into FDA thinking regarding NGS technology and the use of multi-gene panels. The FDA has indicated that a guidance on regulated NGS assays is due in 2014 and has proposed that individual companies request early pre-submission meetings with the Agency to discuss Dx development plans and trial design. It is encouraging that FDA officials have offered at public forums personal opinions that convey the Agency's enthusiasm about the technology and its application for therapy, as well as the recognition of the inevitability of usage of NGS-based tests in public health (focr.org). The FDA has encouraged early and open dialog on the NGS CDx process and has implied that the process, in spite of its complexity, is likely to be facilitated in a manner as similar as possible to that done for existing complex Dx assays.

DIAGNOSTIC DEVELOPMENT CHALLENGE 2 – COMPETITION FROM LDTs

The current environment is one in which NGS-based lab-developed tests are rapidly gaining popularity in the healthcare community and the growing use of NGS-based cancer genome profiling may be pushing the community toward a fast adoption of NGS-based tests. Although there are several sets of guidelines and recommendations (CLIA, CAP, and state guidances) (43, 44) describing the validation and use of existing NGS LDTs, the FDA has indicated that regulation may be necessary to stop the growth of less rigorously validated assays and to reduce the risk to patients. The oncology community's clamor for an information rich NGS Dx is possibly similar to the initial excitement around using microarrays as Dxs, with the goal of having a single comprehensive test that captures a large amount of relevant content. Tests that identify patients that benefit, or not benefit, from certain treatments represent new opportunities and a new market for some companies. Many different types of companies are building research usage only (RUO) cancer panels in the expectation that they could be adopted as LDTs. Other companies are setting up laboratories or expanding their current laboratory capabilities to offer LDT cancer panels and other NGS-based tests (47, 124). The latter represent a significant threat for Dx companies and may make them hesitant to invest heavily in developing an FDA-approved Dx product, especially as less regulated LDTs continue to increase their segment of the Dx market. For example, the recently FDA-approved molecular Dx BRAF V600E test was followed by the development and rapid uptake of cheaper LDTs. FDA recently issued a guidance document (Distribution of IVD Products labeled for Research Use Only or IUO) which may address some of the issues with RUO marketing in particular.

DIAGNOSTIC COMPANY CHALLENGE 3 – LDT VERSUS IVD KIT CONSIDERATIONS

Both LDTs and kit-based Dxs are considered to be *in vitro* Dxs by the FDA, and either can go through the PMA process. Thus, one of the major decisions for NGS-based Dxs developers will be choosing between development of a LDT (currently working under enforcement discretion from FDA regulation) or a commercialized kit-based FDA-approved product. In this context drug companies can choose to partner with "traditional Dx companies" which do not work with a LDT model (they don't have or want a CLIA service lab) or with "Lab-focused Dxs companies" which have a CLIA service lab and that could potentially offer an LDT-based Dx.

Currently, the NGS-based genetic tests on the market are all CLIA/CAP-regulated lab-developed tests (11). To date, none of these tests have been cleared or approved through FDA's stringent pre-marketing review process, which verifies the performance claims of the test. To date only a very small number of molecular genetic tests have FDA approval for marketing as CDxs. Examples of FDA-approved kitted CDxs are the Roche COBAS 4800 test for BRAF V600E mutation detection as a CDx for vemurafenib (Zelboraf) and the Abbott Vysis ALK Break Apart FISH Probe test to identify ALK-positive NSCLC patients for Pfizer's approved NSCLC therapy Xalkori (Crizotinib) (108, 109, 113, 125). There is a separate class of lab-based, FDA-cleared IVDs, e.g., the Agendia MammaPrint assay (126) and the XDx AlloMap assay (127). The largest class of genetic tests is currently unregulated clinical lab-developed tests. Clinical labs are overseen and regulated by the Centers for Medicare and Medicaid Services (CMS) with the CLIA certification process (40). Lab-developed test markets have grown mainly because the FDA approval process is time-consuming and very expensive (117). The extensive clinical validation and design

control requirements expected in FDA-regulated IVD products are deterring many companies from submitting their NGS-based tests for the IVD process. At the same time, valid concerns about the lack of regulatory oversight that allows tremendous variability in test results from LDTs have led to a call for stricter regulation of the LDT (14). The FDA has sought more involvement in LDT regulation for a while now and there is increasing indication that LDT regulation will be on FDA's agenda as evident in FDA's Guidance on Personalized Medicine from October 2013. In 2010, FDA announced plans to expand its regulation to lab-developed genetic tests. This announcement led to heated debate within the industry (117). While this is yet to happen, it may impact the clinical LDT format of NGS assays should they become a specific CDx that requires FDA clearance or approval.

CONCLUDING THOUGHTS

We have provided a summary of the practical challenges to the widespread adoption of NGS-based CTAs and their further development as CDxs. For some challenges we suggested possible remedies that alleviate some of these concerns; for others we framed the relevant questions from a stakeholder's perspective.

It is certain that despite the challenges, in the near future NGS-based Dxs will be a major component of the highly remunerative personalized medicine and Dx industry. What was said about genome sequencing may also be true for clinical NGS-based Dx testing: that we may be overestimating the impact in the short run but we are probably underestimating the impact in the long run (original quote is attributed to renowned futurist Roy Amara). It is a certainty that the healthcare system will be transformed if the technology is embraced and implemented into clinical practice with its full potential. We project that a variety of NGS Dx associated companies or specializations will see exponential growth as they aid the simplification of NGS in the clinic, especially those that offer easy-to-use clinical interpretation interfaces or EMR data incorporation methodologies. It is also foreseeable that clinical NGS will be coupled with methods for minimally invasive monitoring utilizing bio-fluid-based assays instead of traditional tissue biopsies. It is also notable that as pharmaceutical companies and healthcare systems drive clinical NGS into practice, several models for global collaboration between pharmaceutical companies may arise which can help the field of personalized medicine move forward exponentially.

ACKNOWLEDGMENTS

We thank Steven Engel, Ken Emancipator, and Mya Thomae for reading the manuscript and making helpful suggestions. We acknowledge the NGS diagnostic teams at Illumina (Nitin Udar, Naomi O'Grady, and Alex Young) and Life Technologies (Jill Stefanelli and Brian Kelly) for helpful discussions around the CTA and diagnostic aspects of their platforms. We thank Gary Latham, Matt Hawryluk, Nazneen Aziz, Anup Madan, and Seth Crosby for stimulating discussions.

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Conflict of Interest Statement: Applies to all authors: Merck purchases reagents and instruments from Illumina and Life Technologies and owns an Illumina MiSeq instrument and an Ion Torrent Personal Genome Machine. Merck has purchased services from Foundation Medicine using the Foundation One multi-gene NGS panel.

*Received: 19 December 2013; accepted: 28 March 2014; published online: 17 April 2014. Citation: Pant S, Weiner R and Marton MJ (2014) Navigating the rapids: the development of regulated next-generation sequencing-based clinical trial assays and companion diagnostics. Front. Oncol. **4**:78. doi: 10.3389/fonc.2014.00078*
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology.

frontiers in ONCOLOGY

REVIEW ARTICLE
published: 13 June 2014
doi: 10.3389/fonc.2014.00141

Co-development of diagnostic vectors to support targeted therapies and theranostics: essential tools in personalized cancer therapy

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Novel technologies are being developed to improve patient therapy through the identification of targets and surrogate molecular signatures that can help direct appropriate treatment regimens for efficacy and drug safety. This is particularly the case in oncology whereby patient tumor and biofluids are routinely isolated and analyzed for genetic, immunohistochemical, and/or soluble markers to determine if a predictive biomarker signature (i.e., mutated gene product, differentially expressed protein, altered cell surface antigen, etc.) exists as a means for selecting optimal treatment. These biomarkers may be drug-specific targets and/or differentially expressed nucleic acids, proteins, or cell lineage profiles that can directly affect the patient's disease tissue or immune response to a therapeutic regimen. Improvements in diagnostics that can prescreen predictive response biomarker profiles will continue to optimize the ability to enhance patient therapy via molecularly defined disease-specific treatment. Conversely, patients lacking predictive response biomarkers will no longer needlessly be exposed to drugs that are unlikely to provide clinical benefit, thereby enabling patients to pursue other therapeutic options and lowering overall healthcare costs by avoiding futile treatment. While patient molecular profiling offers a powerful tool to direct treatment options, the difficulty in identifying disease-specific targets or predictive biomarker signatures that stratify a significant fraction within a disease indication remains challenging. A goal for drug developers is to identify and implement new strategies that can rapidly enable the development of beneficial disease-specific therapies for broad patient-specific targeting without the need of tedious predictive biomarker discovery and validation efforts, currently a bottleneck for development timelines. Successful strategies may gain an advantage by employing repurposed, less-expensive existing agents while potentially improving the therapeutic activity of novel, target-specific therapies that may otherwise have off-target toxicities or less efficacy in cells exhibiting certain pathways. Here, we discuss the use of co-developing diagnostic-targeting vectors to identify patients whose malignant tissue can specifically uptake a targeted anti-cancer drug vector prior to treatment. Using this system, a patient can be predetermined in real-time as to whether or not their tumor(s) can specifically uptake a drug-linked diagnostic vector, thus inferring the uptake of a similar vector linked to an anti-cancer agent. If tumor-specific uptake is observed, then the patient may be suitable for drug-linked vector therapy and have a higher likelihood of clinical benefit while patients with no tumor uptake should consider other therapeutic options. This approach offers complementary opportunities to rapidly develop broad tumor-specific agents for use in personalized medicine.

Keywords: companion diagnostics, CDx, co-development, TM601, theranostics, nanoparticles, naturally occurring proteins, personalized medicine

PERSONALIZED MEDICINE IN THE ERA OF TARGETED THERAPEUTIC STRATEGIES

Personalized medicine is generally considered as the precise use of drug(s) that can specifically target a patient's diseased tissue. This is typically achieved by using a diagnostic biomarker or biomolecular signature that can predict clinical response in patients before they are treated (1). In the broadest sense, an example of personalized medicine can be found in the therapeutic management of type 1 diabetes. Patients with this condition are identified initially by abnormal blood glucose levels, whereby glucose serves as a biomarker. Once confirmed by follow-up testing, the disease can be managed by drugs capable of modulating active insulin levels.

In more complex diseases such as cancer, an array of genetic and altered gene product expression changes may be required to determine or predict a patient's specific response(s) to therapy. Anti-cancer therapeutic strategies include: (i) binding to a specific

molecular target of an altered pathway or a sequence-specific gene product that in turn results in selective killing of malignant but not normal cells; (ii) inducing a host immune response against malignant cells; and (iii) enhancing specific uptake of an agent(s) in target cells for disease suppression. Based on their chemical or biochemical nature, targeted anti-cancer agents can be classified into small chemical entities (SCE) capable of disrupting cellular processes such as enzymatic reactions, tubulin polymerization and DNA replication; nucleic acids that can specifically bind a gene product involved in tumor growth and metastasis; and cellular- and protein-based therapies that can specifically target tumor-associated cell surface proteins or soluble ligands (2). All these agents exert their pharmacologic activity by specifically suppressing growth and survival in malignant vs. normal cells.

For targeted cancer therapies, it is important that the compound can specifically bind to a gene product (e.g., ligands/receptors, transcription factors, or enzymes) or a molecular target within a pathway unique to a tumor cell or cells located within the tumor microenvironment that support tumor growth. These agents may include cell and protein-based vaccines, peptides, recombinant proteins, antibodies, antibody fragments, nucleic acid, and target-specific SCEs (2). The development of novel SCEs targeting tumor-altered gene products involved in driving the underlying cause of transformation is expanding across the industry as a result of recent approvals of compounds in this class (3). These include the anti BCR–ABL fusion protein tyrosine kinase inhibitor imatinib (4) and the more recently approved translocated ALK inhibitor, crizotinib (5), and mutant BRAF inhibitor, vemurafenib (6). While this class has shown robust clinical activity in patients containing the altered gene product, the low target frequency of the latter two compounds has minimized their utility in the greater patient population. Other classes of targeted therapies include antibody and protein-based agents that can specifically bind cell surface proteins on tumor cells and in turn block or activate receptor signaling, induce programed cell death and/or induce immune-mediated cytotoxicity. In all cases, patients may be prescreened to determine if their tumor expresses an agent-specific molecular target. These examples provide support for the validity of discovering modified disease-specific gene products that can serve as drug targets and be used to prescreen patients via diagnostic platforms capable of identifying those eligible for target-specific therapy.

Other personalized platforms include those that monitor gene expression profiles or soluble markers contained within biofluids (serum, plasma, urine, sputum, or whole cells) that may serve as surrogates for predictive response to a therapeutic regimen. While the example of type 1 diabetes fits into this category, broader discovery approaches are being pursued in oncology. These include biomarker signature profiles within disease tissue that may predict response to certain chemotherapeutic regimens as well as modifier genes that may predict response to a targeted therapy (7). The use of tumor-specific biomarker signature profiles has been widely pursued in breast cancer based on early successful prognostic and therapeutic paradigms that relied on tumor stage and grade, as well as HER2, estrogen (ER), and progesterone (PR) receptor expression status. Subsequent efforts have further refined breast cancer marker profiling to guide best course of therapy (8). This was achieved by development of a molecular signature panel comprising 21 genes, called Oncotype DX® (9), and subsequently MammaPrint™ (10), which comprises a 70 gene set. In both cases, surrogate gene expression profiles are measured to predict a patient's prognosis and guidance for use of existing therapies. More recently, a similar product for patient prognosis in colorectal cancer (OncoDX) has been developed (11). While these molecular signatures have value in predicting an individual's prognosis, they cannot predict potential clinical responses after specific targeted therapy. Despite their success and widespread use in breast or colorectal cancer for clinical follow-up after initial diagnosis, the generation and validation of these gene product signatures has taken a significant amount of time and effort before achieving clinical utility in managing personalized treatment for cancer patients.

There are currently 19 FDA approved companion diagnostic (CDx) assays, 18 of which are approved in oncology (**Table 1**). Ten of the 18 CDx assays are various qualitative assays for detecting HER2 expression or amplification in breast cancer; 2 are qualitative assays for BRAF V600E mutations; along with qualitative assays for ALK translocations, KRAS mutations and c-KIT; and 3 qualitative assays for EGFR mutations. The relative paucity of FDA approved CDx assays might reflect the difficulty and complexity in requirements for approval of such assays but might also be a reflection of a lack of in-depth knowledge of the underlying biology of cancer and/or the drug target interaction. Further, it is interesting to note that only 3 of the 18 (17%) oncology CDx assays are quantitative in format. While quantitative assays are not necessarily required, this undoubtedly speaks to the differences in regulatory requirements for quantitative relative to qualitative assays, at least with respect to CDx assays.

Interestingly, many more molecular diagnostic (Dx) assays are in drug labels (**Table 2**) – not as companion diagnostics but recommended or even required prior to prescribing therapy or for therapeutic monitoring – and some of these have been cleared by the FDA through the 510(k) process (**Table 3**). Complex pharmacogenomics signatures such as Oncotype DX® and even BRCA mutation analyses (12) are routinely used in clinical oncology but are performed under the CLIA regulation (Clinical Laboratory Improvement Amendments of 1988) and are classified as Laboratory Developed Tests (LDTs). This class of Dx does not go through the rigor of regulatory submissions such as 510(k) or PMA (Pre-Market Approval; a CDx requires a PMA) nor the post-marketing requirements of such assays. The FDA is currently reviewing and is expected to make recommendations in the near future regarding the oversight of LDTs, which may (significantly) change the present landscape.

ANTIBODY-DRUG CONJUGATES AND RADIOIMMUNOTHERAPIES

Recent clinical success of monoclonal antibody (mAb) drug conjugates (ADCs) has spurred the field of highly toxic chemotherapeutic drugs for targeted therapy. The development of ADCs offers dual benefits: the ability to preselect patients whose disease expresses the target antigen for tumor-specific delivery and the opportunity to deliver highly toxic (novel or repurposed) compounds to antigen positive tumors while avoiding toxic off-target effects commonly found with non-targeted SCEs or radionuclides

Table 1 | FDA approved companion diagnostics (CDx).

Drug trade name (generic name)	Device trade name	Device manufacturer	Approved	Technology/indication
Erbitux (cetuximab)	therascreen KRAS RGQ PCR Kit	Qiagen	2012	Qualitative RT-PCR/CRC
Erbitux (cetuximab); Vectibix (panitumumab)	DAKO EGFR PharmDx Kit	Dako	2006	Qualitative IHC/CRC
Exjade (deferasirox)	Ferriscan	Resonance Health Analysis Services	2013	FerriScan R2-MRI/thalassemia
Gilotrif (afatinib)	Therascreen EGFR RGQ PCR Kit	Qiagen	2013	Qualitative RT-PCR/NSCLC
Gleevec/glivec (imatinib mesylate)	Dako C-KIT PharmDx	Dako	2012	Qualitative IHC/GIST
Herceptin (trastuzumab)	INFORM HER2/neu	Ventana Medical Systems	2000	Qualitative FISH/breast cancer
	PathVysion HER2 DNA Probe Kit	Abbott Molecular	2013	Qualitative FISH/breast cancer
	PATHWAY anti-HER2/neu	Ventana Medical Systems	2013	Semi-quantitative IHC/breast cancer
	InSite HER2/neu Kit	BioGenex Laboratories	2005	Semi-quantitative IHC/breast cancer
	SPOT-Light HER2 CISH Kit	Life Technologies	2012	Quantitative CISH/breast cancer
	Bond Oracle Her2 IHC System	Leica Biosystems	2012	Semi-quantitative IHC/breast cancer
	HER2 CISH PharmDx Kit	Dako	2013	Quantitative ISH/breast cancer
	INFORM HER2 Dual ISH DNA Probe Cocktail	Ventana Medical Systems	2013	Two-color ISH/breast cancer
Herceptin (trastuzumab) Perjeta (pertuzumab)	HercepTest	Dako	2013	Semi-quantitative IHC/breast cancer, metastatic gastric, or gastroesophageal junction adenocarcinoma
KADCYLA (ado-trastuzumab emtansine)	HER2 FISH PharmDx Kit	Dako	2013	Quantitative FISH/breast cancer, metastatic gastric, or gastroesophageal junction adenocarcinoma
Mekinist (tramatenib); Tafinlar (dabrafenib)	THxID™ BRAF Kit	bioMérieux	2013	Qualitative RT-PCR/melanoma
Tarceva (erlotinib)	Cobas EGFR Mutation Test	Roche Molecular Systems	2013	Qualitative RT-PCR/NSCLC
Xalkori (crizotinib)	Vysis ALK Break Apart FISH Probe Kit	Abbott Molecular	2013	Qualitative FISH/NSCLC
Zelboraf (vemurafenib)	Cobas 4800 BRAF V600 Mutation Test	Roche Molecular Systems	2013	Qualitative RT-PCR/melanoma

Source: http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm.

(hereon referred to as cytotoxins) (13). Enhanced technologies that enable robust linkage of a targeting agent to a cytotoxin such as radionuclides, chemotherapeutic SCEs, and gene silencing nucleic acids has led to the establishment of a wide array of next generation targeted therapies (14). The targeting moieties themselves have varied from full-length antibodies to recombinant proteins, small polypeptides, and nanoparticles (NPs). A diversity of linkage chemistries that allow conjugation of a cytotoxin to the targeting agent have been implemented depending upon where in the tissue it is most desirable to have the cytotoxin delivered and, if required, liberated from the targeting agent. With the early success of antibody–cytotoxin conjugates using radionuclides (referred to as radioimmunotherapy, RIT), such as yttrium-90 (^{90}Y)-labeled-ibritumomab tiuxetan (15) and iodine-131 (^{131}I)-labeled tositumomab (16) in treating refractory lymphoma, as well as the recently approved ADC trastuzumab-DM1 (T-DM1, Kadcyla®) (17), and brentuximab vedotin (SGN35, Adcetris®) (18), significant progress in personalized medicine has been attained (19). Part of this advancement is due to the improved therapeutic activity over the parental agents (the cytotoxic or targeting agent alone) resulting in a better clinical outcome while minimizing toxicity. Rituximab is a chimeric mouse–human IgG1 mAb directed to the CD20 cell surface protein and approved for treatment of B-cell lymphomas. ^{90}Y-labeled-ibritumomab

Table 2 | Molecular diagnostics (Dx) in US drug labels.

Drug	HUGO symbol	Referenced subgroup	Labeling sections
Ado-trastuzumab emtansine	ERBB2	HER2 protein overexpression or gene amplification positive	Indications and usage, warnings and precautions, adverse reactions, clinical pharmacology, clinical studies
Afatinib	EGFR	EGFR exon 19 deletion or exon 21 substitution (L858R) mutation positive	Indications and usage, dosage and administration, adverse reactions, clinical pharmacology, clinical studies, patient counseling information
Anastrozole	ESR1, PGR	Hormone receptor positive	Indications and usage, clinical pharmacology, clinical studies
Arsenic trioxide	PML/RARα	PML/RARα [t(15;17)] gene expression positive	Boxed warning, clinical pharmacology, indications and usage, warnings
Bosutinib	BCR/ABL1	Philadelphia chromosome [t(9;22)] positive	Indications and usage, adverse reactions, clinical studies
Brentuximab vedotin	TNFRSF8	CD30 positive	Indications and usage, description, clinical pharmacology
Busulfan	Philadelphia chromosome	Ph chromosome negative	Clinical studies
Capecitabine	DPYD	DPD deficient	Contraindications, warnings and precautions, patient information
Cetuximab	EGFR	EGFR protein expression positive	Indications and usage, warnings and precautions, description, clinical pharmacology, clinical studies
	KRAS	KRAS codon 12 and 13 mutation negative	Indications and usage, dosage and administration, warnings and precautions, adverse reactions, clinical pharmacology, clinical studies
Cisplatin	TPMT	TPMT intermediate or poor metabolizers	Clinical pharmacology, warnings, precautions
Crizotinib	ALK	ALK gene rearrangement positive	Indications and usage, dosage and administration, drug interactions, warnings and precautions, adverse reactions, clinical pharmacology, clinical studies
Dabrafenib	BRAF	BRAF V600E mutation positive	Indications and usage, dosage and administration, warnings and precautions, clinical pharmacology, clinical studies, patient counseling information
	G6PD	G6PD deficient	Warnings and precautions, adverse reactions, patient counseling information
Dasatinib	BCR/ABL1	Philadelphia chromosome [t(9;22)] positive; T315I mutation posit	Indications and usage, clinical studies, patient counseling information
Denileukin diftitox	IL2RA	CD25 antigen positive	Indications and usage, warnings and precautions, clinical studies
Erlotinib	EGFR	EGFR protein expression positive EGFR exon 19 deletion or exon 21 substitution (L858R) positive	Clinical pharmacology Indications and usage, dosage and administration, clinical pharmacology, clinical studies
Everolimus	ERBB2	HER2 protein overexpression negative	Indications and usage, boxed warning, adverse reactions, use in specific populations, clinical pharmacology, clinical studies
Everolimus	ESR1	Estrogen receptor positive	Clinical pharmacology, clinical studies
Exemestane	ESR1	Estrogen receptor positive	Indications and usage, dosage and administration, clinical studies, clinical pharmacology

(Continued)

Table 2 | Continued

Drug	HUGO symbol	Referenced subgroup	Labeling sections
Fluorouracil	DPYD	DPD deficient	Warnings
Fulvestrant	ESR1	Estrogen receptor positive	Indications and usage, clinical pharmacology, clinical studies, patient counseling information
Ibritumomab tiuxetan	MS4A1	CD20 positive	Indications and usage, clinical pharmacology, description
Imatinib	KIT	c-KIT D816V mutation negative	Indications and usage, dosage and administration clinical pharmacology, clinical studies
	BCR/ABL1	Philadelphia chromosome [t(9;22)] positive	
	PDGFRβ	PDGFR gene rearrangement positive	
	FIP1L1/PDGFRα	FIP1L1/PDGFRα fusion kinase (or CHIC2 deletion) positive	Indications and usage, dosage and administration, clinical studies
Irinotecan	UGT1A1	UGT1A1*28 allele carriers	Dosage and administration, warnings, clinical pharmacology
Lapatinib	ERBB2	HER2 protein overexpression positive	Indications and usage, clinical pharmacology, patient counseling information
Letrozole	ESR1, PGR	Hormone receptor positive	Indications and usage, adverse reactions, clinical studies, clinical pharmacology
Mercaptopurine	TPMT	TPMT intermediate or poor metabolizers	Dosage and administration, contraindications, precautions, adverse reactions, clinical pharmacology
Nilotinib	BCR/ABL1	Philadelphia chromosome [t(9;22)] positive	Indications and usage, patient counseling information
Nilotinib	UGT1A1	UGT1A1*28 allele homozygotes	Warnings and precautions, clinical pharmacology
Ofatumumab	MS4A1	CD20 positive	Indications and usage, clinical pharmacology
Omacetaxine	BCR/ABL1	BCR-ABL T315I	Clinical pharmacology
Panitumumab	EGFR	EGFR protein expression positive	Indications and usage, warnings and precautions, clinical pharmacology, clinical studies
	KRAS	KRAS codon 12 and 13 mutation negative	Indications and usage, clinical pharmacology, clinical studies
Pazopanib	UGT1A1	(TA)7/(TA)7 genotype (UGT1A1*28/*28)	Clinical pharmacology, warnings and precautions
Pertuzumab	ERBB2	HER2 protein overexpression positive	Indications and usage, warnings and precautions, adverse reactions, clinical studies, clinical pharmacology
Ponatinib	BCR/ABL1	Philadelphia chromosome [t(9;22)] positive, BCR–ABL T315I mutation	Indications and usage, warnings and precautions, adverse reactions, use in specific populations, clinical pharmacology, clinical studies
Rasburicase	G6PD	G6PD deficient	Boxed warning, contraindications
Rituximab	MS4A1	CD20 positive	Indication and usage, clinical pharmacology, description, precautions

(Continued)

Table 2 | Continued

Drug	HUGO symbol	Referenced subgroup	Labeling sections
Tamoxifen	ESR1, PGR F5 F2	Hormone receptor positive Factor V Leiden carriers Prothrombin mutation G20210A	Indications and usage, precautions, medication guide Warnings
Thioguanine	TPMT	TPMT poor metabolizer	Dosage and administration, precautions, warnings
Tositumomab	MS4A1	CD20 antigen positive	Indications and usage, clinical pharmacology
Trametinib	BRAF	BRAF V600E/K mutation positive	Indications and usage, dosage and administration, adverse reactions, clinical pharmacology, clinical studies, patient counseling information
Trastuzumab	ERBB2	HER2 protein overexpression positive	Indications and usage, warnings and precautions, clinical pharmacology, clinical studies
Tretinoin	PML/RARA	PML/RARα [t(15;17)] gene expression positive	Clinical studies, indications and usage, warnings
Vemurafenib	BRAF	BRAF V600E mutation positive	Indications and usage, warning and precautions, clinical pharmacology, clinical studies, patient counseling information

Source: http:// www.fda.gov/ Drugs/ ScienceResearch/ ResearchAreas/ Pharmacogenetics/ ucm083378.htm

tiuxetan and I^{131}-labeled tositumomab, both of which also target CD20, showed statistically significant clinical responses in patients as compared to rituximab or chemotherapy alone and were approved for use in rituximab-refractory patients. Unfortunately, the application of these RITs in clinical practice has been limited by the complexity of handling β-emitting radionuclide-labeled mAbs before and after patient treatment. These limitations have fostered the generation of alternate molecules, including alpha emitting RITs (20) as well as non-radioactive cytotoxins that can be more practically conjugated to mAb, protein, or peptide-based targeting agents, without affecting their pharmacokinetic or pharmacodynamic properties (i.e., diminished ability to maximally and specifically access its target expressed by the diseased tissue). While therapeutic improvements have been reported in cancers using RITs and ADCs vs. non-conjugated agents, not all patients treated with RIT or ADC agents have shown enhanced benefit, suggesting diagnostic opportunities for improving the therapeutic use of conjugates (21). **Table 4** provides an overview of approved ADCs and RITs.

As indicated above, continued improvement and development of targeted therapies, using ADCs, RITs, or other technologies, is required but not sufficient to realize the maximal therapeutic potential of personalized medicine. Tailoring of a therapy to an individual's cancer requires knowledge of the underlying biology of that cancer and may involve utilizing surrogate molecular signatures, drug target expression profiles, and/or degree of targeted conjugate uptake for predicting patient response. The heterogeneity described for individual tumors (22) only adds to the complexity of defining the biomolecular characteristics of a patient's malignant tissue and selecting a therapy, or combination of therapies, most likely to be effective for the individual patient. In turn, such tumor heterogeneity adds to the complexity of development of the requisite surrogate Dx or CDx that is required to maximize the benefit of targeted therapies. It is generally accepted that the more complex the diagnostic platform, the more intricate is the regulatory path for approval, especially if the diagnostic is a CDx. A CDx is considered high risk by most regulatory authorities as it specifically dictates therapeutic intervention, in contrast to Oncotype Dx, MammaPrint, or OncoDX type diagnostic tools that merely supply information relative to prognosis and guide the therapeutic intervention. The development, analytical, and clinical validation of complex multi-marker diagnostic biomarker signatures is both time consuming and expensive. In addition, the alignment of therapeutic-diagnostic development timelines is challenging at best, especially if such signatures are not discovered until *post hoc* analysis of Phase 2 clinical trials. The recent clearance by the US FDA of next generation sequencing (NGS) instrumentation for cystic fibrosis is an important step for the use of new technologies to support complex assay developments, particularly as they relate to oncology where such complex signatures are likely required (23). However, as noted, biomarker signatures for predicting response to a given therapy may not simply involve gene expression or mutation profiles but, rather, complex gene product expression profiles.

TARGETED CYTOTOXIC AGENTS – TCAs

Despite the successful demonstration that targeted cytotoxic agents (TCAs), such as ADCs and RITs, can provide added clinical benefit for certain cancers, a number of challenges still remain for their clinical success across a broad spectrum of cancer indications. The effectiveness of targeted cytotoxin conjugates depends in part on the inherent features of the conjugate used. Some of the

Table 3 | FDA 510(k) cleared molecular diagnostics (Dx).

Disease state	Device	Year	Device manufacturer	Comments
AML	Vysis EGR1 FISH Probe Kit	2011	Abbott Molecular	Deletions in EGR1; bone marrow specimens; aid in prognosis
B-cell CLL	Vysis CLL FISH Probe Kit	2011	Vysis	Deletions in TP53, ATM, and D1I3S319 and gain in D1I2Z3; peripheral blood; aid in prognosis
	CEP 12 DNA Probe	1997	Vysis	FISH; specific for centromere 12; peripheral blood; prognosis
Bladder cancer	Vysis UroVysion Bladder Cancer Recurrence Kit	2004	Vysis	Aneuploidy of chromosomes 3, 7, 17 and loss of 9p21 locus; urine specimens; TCC; monitor recurrence
Breast cancer	MammaPrint	2011	Agendia	Gene expression profile; fresh frozen tissue; assess risk for distant metastasis and prognosis
	GeneSearch Breast Lymph Node (BLN) Test Kit	2009	Veridex	Gene expression panel; metastasis in lymph nodes; aids in the decision to excise additional lymph nodes and staging
	Dako TOP2A FISH PharmDx Kit	2012	Dako	FISH to detect copy number changes of TOP2A; FFPE; prognosis in high risk breast cancer patients
Cystic fibrosis	eSensor CF Genotyping Test	2009	Osmetech Molecular Diagnostics	Detects a panel of mutations and variants in CFTR; genomic DNA; genetic carrier screening
	xTAG Cystic Fibrosis 60 Kit v2	2009	Luminex Molecular Diagnostics	Detects and identifies a panel of mutations and variants the CFTR; genetic carrier and newborn screening
Prostate cancer	NADiA ProsVue	2011	Iris Molecular Diagnostics	Determines rate of change of total PSA; serum; an aid in identifying those patients at reduced risk for recurrence of prostate cancer
	PROGENSA PCA3 Assay	2012	Gen-Probe	PCA3 and PSA RNA ratio; urine; aids physicians in determining the need for repeat prostate biopsies in men who have had a previous negative biopsy
Tissue of origin	Pathwork Tissue of Origin Test Kit – FFPE	2012	Pathwork Diagnostics	Compares RNA expression patterns in a patient's FFPE tumor with those in a database; tissue; aid in determining origin of cancer
	Pathwork Tissue of Origin Test	2008	Pathwork Diagnostics	Compares RNA expression patterns in a patient's fresh/frozen tumor with those in a database; tissue; aid in determining origin of cancer

Source: http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm330711.htm

TCA properties that can be optimized include: (1) tumor recognition and penetration; (2) serum half-life to minimize liberation of the cytotoxin in serum that may result in off-target effects; (3) targeting epitopes on a cell surface antigen that can support maximal conjugate internalization; (4) ability of the targeting agent to retain its target specificity in the conjugated form; and (5) large-scale conjugation of the cytotoxin to the targeting moiety for GMP manufacturing at a reasonable cost-of-goods. Smaller molecular weight targeting conjugates that employ antibody fragment or peptide platforms offer the opportunity to improve TCA tumor penetration (21), enhance binding specificities (24) and internalization (25), as well as lower serum half-lives to avoid prolonged circulation (26). Furthermore, smaller sized TCAs offer the ability to employ alternative manufacturing approaches to minimize cost-of-goods in contrast to mammalian cell fermentation that is required for manufacturing of full-length monoclonal antibodies. While antibody and antibody fragment conjugates appear to offer additional benefits for developing disease-specific therapies, the limited frequency in which a cell surface target is strictly expressed across heterogeneous disease vs. normal tissue remains a major drawback. In cancer, several cell surface targets have been identified that appear to be tumor-specific but the frequency of expression is quite variable from one tumor type to another thereby limiting the breadth by which an approved TCA can be used across different cancer indications (27). Furthermore, recent studies have demonstrated that the expression levels and/or distribution of cell surface targets on tumor cells or tumor-associated stromal cells can vary within the same specimen (28). Hence, the development of high affinity and high specificity targeting agents to maximize tumor recognition in cases of low and heterogeneous target expression is needed. The number of broadly expressed molecular targets that are present on a diseased cell and not on normal tissues that can be selectively targeted by a TCA is low. Nevertheless, several disease-specific antigens have been identified as a result of epigenetic mechanisms, alternative splicing, gene rearrangement, and overexpression that

Table 4 | Examples of approved antibody-drug conjugates (ADCs) and radioimmunotherapeutics (RIT's) in oncology.

Trade name (generic)	Manufacturer	Target	Conjugate	Approved	Indication	Comments
ANTIBODY-DRUG CONJUGATES (ADCs)						
Mylotarg Gemtuzumab ozogamicin	Pfizer/Wyeth	CD33	Calecheamicin	2001	Recurrent AML (age 60+)	Voluntarily withdrawn in 2010, due to product safety issues and lack of clinical benefit
Adcetris Brentuximab vedotin	Seattle Genetics	CD30	Mono-methyl auristatin E (MMAE)	2011	Refractory Hodgkin's lymphoma Refractory systemic anaplastic large cell lymphoma	
Kadcyla Trastuzumab emtansine	Genentech/ Roche	Her2/ neu	Maytansinoid DM1	2013	HER2-positive metastatic breast cancer	Approved for patients who have received prior treatment with Herceptin® (trastuzumab) and a taxane chemotherapy
Trade name (generic)	**Manufacturer**	**Target**	**Isotope**	**Approved**	**Indication**	**Comments**
RADIOIMMUNOTHERAPEUTICS (RITs)						
Zevalin Ibritumomab tiuxetan	Biogen-Idec/ Spectrum pharmaceuticals	CD20	^{90}Y	2002	Recurrent, low-grade or follicular B-cell non-Hodgkin's lymphoma	
Bexxar Iodine (131I) tositumomab	Corixa/GSK	CD20	^{131}I	2003	CD20 positive, follicular NHL, refractory to rituximab and relapsed following chemotherapy	Manufacture discontinued in 2014 due to poor sales

support the potential use of this class for maximizing the therapeutic potential of targeted agents (27). As efforts continue across the industry to identify more disease-specific targets via a variety of genomic and proteomic discovery approaches (discussed below), more broadly expressed disease-associated targets and disease targeting agents have been identified from the screening of naturally occurring pathogenic proteins, intra-protein domains, and NPs scaffolds (29–31). These platforms offer the potential to employ theranostics: the co-development of a TCA along with the same targeting vector linked to a diagnostic agent to determine effective targeting and patient selection (32). Moreover, the use of TCA formats enables the potential repurposing of pharmacologically defined cytotoxic agents on the market, which may lead to faster development timelines of TCA by leveraging prior clinical experience, or the salvaging of compounds that showed anti-tumor activity in clinical trials but failed due to off-target toxicities. One should also bear in mind that, while the expression of the target is required, it may not be sufficient for long-lasting responses. In fact, due to the inherent heterogeneity of tumors and potential escape mechanisms [as seen for example with BRAF inhibitors (33)], theranostics and the TCA strategy in general would likely benefit from being combined with other drugs that have different mode of action and/or target.

NANOPARTICLES AND APTAMERS

Over the past two decades, the use of NPs has shown promise in delivering therapeutic drugs to malignant cells. Early NP-derived agents were primarily designed by optimizing particle size, chemical composition (lipids, silica, nucleic acids), and charge in an attempt to deliver tumor-specificity (34). Next generation NP technologies incorporated the use of disease-specific ligands, such as antibodies and aptamers, which could bind to disease-associated cell surface receptors and deliver therapeutic SCEs. Unfortunately, as mentioned above, the discovery of widely expressed disease-specific receptors that can mediate robust internalization are infrequent. More recently, aptamer-bound NPs have been found to be useful in delivering cytotoxic agents to cancers by targeting disease-specific epitopes on cell surface tumor antigens (35). Peptide aptamers are combinatorial protein molecules usually consisting of short peptides inserted within a scaffold protein resulting in conformational assortment that creates a target-binding diversity. Nucleic acid-based aptamers can achieve similar levels of conformational diversity and target specificity as peptide-based aptamers. Since nucleic acids are also being explored as NP to carry, deliver, and release chemotherapeutic agents, they may represent unique building blocks for both aptamers and NPs manufacturing. The use of aptamers expands the ability to identify subtle differences in the topographical structure of cell surface tumor

antigen motifs that are not as easily recognized by traditional proteomic platforms. In light of their size, aptamer-guided NPs have been further engineered as theranostics, whereby the NP contains the targeting aptamer, a cytotoxic agent and an imaging agent that can monitor tumor uptake directly in the patient (36). Patients showing tumor-specific uptake are then deemed suitable for NP-cytotoxic therapy while those that do not can pursue other therapeutic options. Again, despite these promising results, challenges still remain in aptamer-guided NP theranostics including non-specific NP tissue binding, systemic stability, GMP manufacturing, and broad-based application to multiple cancer types. It is worth noting that the successful development of targeted NPs will be demonstrated through a combination of target specificity, a high tumor-to-normal tissue ratio and affinity that will enable the agent to "find and bind" low target expression to deliver their cytotoxic payload. Similarly, these properties are required for their use in diagnostic modalities including patient selection and monitoring of therapeutic efficacy. **Table 5** contains a list of marketed and clinical stage NPs being developed for oncology.

NATURAL AGENTS TARGETING TUMORS

Several natural agents are able to target differentially expressed or conformation-specific cell surface antigens that are not easily identified by nucleic acid or proteomic analyses nor are easily targeted using traditional protein/antibody approaches. In particular, proteins, toxins or metabolites contained within plants, insects, arthropods, reptiles, viruses, and bacteria have yielded a number of agents capable of binding to specific host cell surface and intracellular proteins as a means to defend against predators and/or suppress their immune system as well as paralyze or even kill their prey (37, 38). Biochemical studies using natural agents from these sources have found them to have disparate activities. These include those that bind and are retained on the cell surface to suppress the activity of enzymes and channels while others have been shown to internalize upon binding to cell type-specific cell surface proteins to suppress intracellular functions. Naturally occurring polypeptides (NOP) from these sources include the following agents: vacuolating toxin A (VacA), which enters human cells via sphingomyelin (39); hepatitis C viral coat protein, which enters cells via claudin-1 (40); *Clostridium perfringens* enterotoxin, which binds to claudin-4 and causes cytotoxicity in cancer cells (41); crotamine, a toxin from rattlesnake venom that enters cells via heparin sulfate proteoglycans (42); and cholorotoxin, which binds to activated epithelial cells and internalizes via the annexin A2 complex (43, 44). Upon further experimental validation of tumor selectivity, any of these agents may serve as potential targeting moieties in the context of a TCA and could also be incorporated into theranostics platforms.

A critical factor for a therapeutic conjugate to provide clinical benefit is the ability to be systemically maintained at a certain molar level in order for the drug to effectively reach the target cells and accumulate at a concentration sufficient for the cytotoxin to exert its pharmacologic effect. Potential drawbacks of using NPs are their relative short serum and intracellular half-lives making extensive dosing and formulation studies critical for their success (45). Alternatively, NOPs have been selected by nature for their ability to impact cellular targets and maintain their function upon exposure. Moreover, their structures have evolved to withstand systemic degradation and immune responses by the host's serum proteolytic and host defense systems. These features along with

Table 5 | Examples of clinical stage nanoparticles and naturally occurring proteins in development for oncology.

Organization	Compound name	Compound description	Target/active agent	Development stage
Alnylam Pharmaceuticals	ALN-VSP	Liposomal based nanoparticle containing siRNA	KSD and VEGF/siRNA	Phase 1
BIND Biosciences	Bind-014	Polylactide–polyethylene glycol biopolymer nanoparticle containing a chemotoxin and targeting ligand	PSMA/docetaxel	Phase 2
Celgene	Nab-paclitaxel	Albumin based nanoparticle	Paclitaxel	Approved (Abraxane®)
Cerulean Pharma	CRLX-101	Cyclodextrin-based nanoparticle encapsulating a chemotoxin	Camptothecin	Phase 1/2
Janssen Pharmaceuticals	Pegylated liposomal doxorubicin	Pegylated liposomal nanoparticle containing a chemotoxin	Doxorubicin	Approved (Doxil®)
Morphotek	TM601	36 Amino acid peptide from scorpion venom that binds transformed cells and tumor endothelial cells via annexin A2 complex	Annexin A2 complex	Phase 1 (naked peptide format)
University of Illinois at Chicago	NSC745104	28 Amino acid fragment of the protein cupredoxin azurin from pseudomonas aeruginosa that increases intracellular p53 concentrations	p53	Phase 1

KSD, kinesin spindle protein; PSMA, prostate-specific membrane antigen; VEGF, vascular endothelial growth factor.

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Monoclonal antibodies, antibody fragments, NPs, or NOPs may be armed with payloads and deployed in a leveraged theranostic strategy through the clever use of chemical linkers or couplers. One can envision three basic configurations for a leveraged theranostic compound: (1) one payload for therapy and one for diagnostics, with these payloads conjugated on different batches of the same targeting molecule; this configuration involves a single manufacturing process for the targeting moiety, and possibly one process for the linker attachment, but two separate processes for the conjugation of the two payload types; (2) one payload for therapy and one for diagnostics co-conjugated on the same targeting moiety; this implies a single compound and manufacturing process and represents a more ideal scenario; and finally (3) a single payload that can be cytotoxic as well as used for tumor uptake monitoring; this configuration allows for a single compound, manufacturing and conjugation process represents the most ideal scenario. An example of the first configuration (**Figure 2A**) is offered by the use of specific radionuclides. A radionuclide could be optimal for cytotoxicity but suboptimal for imaging, or vice versa. However, even by using currently available radionuclides, a single targeting agent such as CTX could be "functionalized" using a single chelator (hence a single manufacturing process for the targeting, functionalized moiety), and conjugated to Indium-111 for patient selection and Yttrium-90 for delivering cytotoxicity to the tumor by using two separate conjugation processes. Indium-111, while an excellent imaging agent, is not useful for therapy due to its low tissue penetration characteristic. These properties are reversed in Yttrium-90. Other radionucleotide pairs could be selected to satisfy the desired pharmacological as well as pharmacodynamic properties of the theranostic agent being pursued.

In the second configuration (**Figure 2B**), one where both payloads are co-attached on the same targeting molecule, one needs to be mindful of stereochemical interferences. For example, multiple payloads can disrupt the tumor cell binding activity of small targeting peptides such as CTX (36 amino acids). Structure–activity relationship analysis would need to be conducted to identify the best chemistry and attachment sites on both the targeting and payload molecules. NPs inherently offer the opportunity to carry multiple payloads to the tumor, including cytotoxins and diagnostic agents, but may not be sufficiently tumor-specific unless coupled with a targeting moiety. Another challenge using multiple payloads could be achieving a defined chemical homogeneity necessary for regulatory approval using a cost-effective manufacturing process.

The third configuration type (**Figure 2C**) has been achieved over the past several years by employing iodine-131, which, as noted above, suffers from the complexity of its handling. Therefore, this configuration could be improved by the selection of optimized radionuclides, their improved manufacturing processes, storage, and handling procedures, and by more sensitive whole-body radio-imaging devices. A new candidate for this mono-payload theranostic strategy is lutetium-177 (51). This radionuclide is a medium-energy β-emitter with a maximal tissue penetration of 2 mm, hence capable of delivering its cytotoxic energy through several cell layers, while potentially having less off-target toxicity than yttrium-90 (12 mm penetration range). Lutetium-177 also emits low-energy γ-rays allowing both imaging and dosimetry (quantitation of delivered or residual dose). Mono-payload, radiolabeled compounds could be used theranostically, whereby: (i) low, diagnostic (non-therapeutic) doses are used for initial assessment of *in vivo* targeting; (ii) sub-therapeutic doses are administered for dosimetry, allowing precise dose selection, and for monitoring potential toxic effect; and (iii) higher, therapeutic doses are administered to continue to monitor toxicity in conjunction with tumor burden (efficacy, acquired resistance) and tumor uptake (disease modifications, such as loss of target). This strategy is already being implemented when using BEXXAR®, an iodine-131-labeled antibody targeting CD20 positive B-lymphoma cells. Using dosimetry, physicians can use a low dose (5 mCi) and directly measure this TCA clearance rate. Patients with high tumor burden, splenomegaly, or bone marrow involvement tend to have faster clearance. Hence, the therapeutic dose (up to 90 mCi) can be prospectively individualized by using an equation (52).

By allowing patient selection and efficacy as well as toxicity monitoring, the potential success of pivotal trials using these theranostic strategies will allow the technological advancement and clinical benefit improvement of personalized medicine. **Table 6**

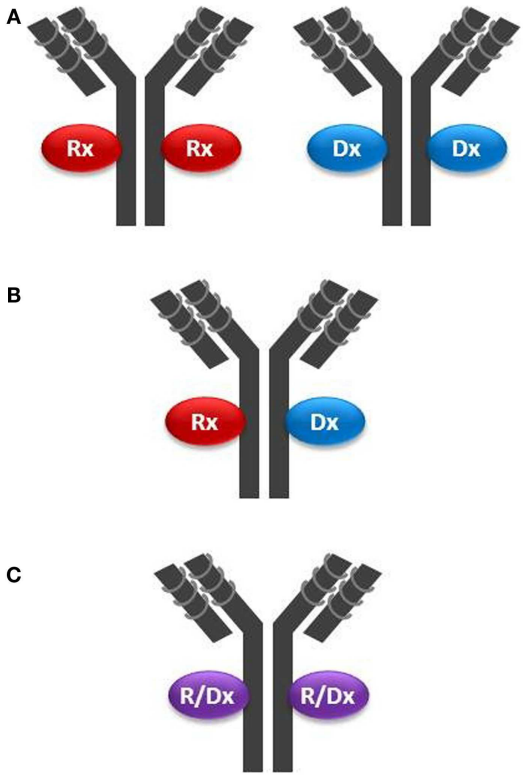

FIGURE 2 | Configurations of theranostic agents: (A) two separate batches of the same targeting moiety (in this example a mAb) are conjugated with either a therapeutic agent (Rx) such as a cytotoxin, or a diagnostic agent (Dx) such as a radionuclide; (B) the same batch of a mAb is conjugated with *both* Rx and Dx agents on the same targeting molecule; and (C) the same batch of a mAb is conjugated with an agent having both Rx and Dx properties (R/Dx), such as Lutetium-177, on the same targeting molecule.

Table 6 | Clinical stage theranostics.

Organization	Rx compound	Dx compound	Targeting moiety	Target	Configuration type	Development stage
Endocyte	Vinca alkaloid	Technetium-99m	Folate	Folate receptors	Figure 2A	Phase 3
Morphotek	Iodine-131	Iodine-131	Chlorotoxin	Annexin A2	Figure 2C	Phase 2
GlaxoSmithKline	Iodine-131	Iodine-131	Tositumomab (murine IgG2a)	CD20	Figure 2C	Approved (Bexxar®)
Institut Jules Bordet	Lutetium-177	Gallium-68	Octreotide (somatostatin analog)	Somatostatin receptor	Figure 2A	Phase 2
Peregrine	Neutralizing mAb	F(ab')2-indium-124	Bavituximab	Phosphatidylserine	Figure 2A[a]	Phase 1[b]/Phase 3[c]
Memorial Sloan-Kettering Cancer Center	Iodine-131	indium-124	8H9 (murine IgG1)	B7-H3	Figure 2A	Phase 1
University Medical Centre Groningen	Neutralizing mAb	Zirconium-89	Trastuzumab	HER2	Figure 2A[a]	Phase 1/2
Institut Jules Bordet/Roche	Maytansine	Zirconium-89	Trastuzumab	HER2	Figure 2A	Phase 2
Areva Med LLC	Lead-212	Lead-212	Trastuzumab	HER2	Figure 2C	Phase 1

[a] *Rx compound is a naked chimera IgG with target-neutralizing activity;*
[b] *Dx compound;*
[c] *Rx compound.*

contains a list of clinical stage theranostics being developed for oncology.

CONCLUSION AND FUTURE DIRECTIONS

The use of personalized medicine has many attributes that make the practice invaluable to patients, the pharmaceutical industry, and the healthcare system. The ability to predefine patients with a high likelihood to respond to a given therapy will provide benefit to all parties. For patients, the ability to predict response will improve therapeutic outcome while avoiding unnecessary treatment with ineffective, potentially toxic drugs and thereby lead to a better quality of life, if not a cure. For the pharmaceutical industry, predictive biomarkers (i.e., informative CDx) may improve the probability of success that a drug will provide meaningful clinical results in trials leading to higher approval rates by regulatory authorities and value-creation for the industry and patients alike. For the healthcare system, the ability to avoid futile, potentially toxic therapies will reduce not only drug costs but overall healthcare costs and potentially improve patient health by identifying agents that have a higher probability of success in treating their specific disease. While these attributes are compelling, the ability to implement platforms to support personalized medicine remains challenging. Attempts to identify disease-specific targets or molecular signatures that can provide predictive response outcomes are ongoing across the pharmaceutical industry and academia alike for many indications. While a few successful examples have been achieved, the majority of development programs are handicapped by the paucity of targets associated with disease as well as the time and effort required to validate molecular signatures that can unequivocally and reproducibly predict patient response to non-targeted SCEs.

As the industry refines its technologies and methods to improve upon personalized medicine, a few platforms exist today that may support this initiative in real-time clinical trials. Of particular note is the use of NPs and NOPs that can be conjugated to a therapeutic agent to improve disease-specific uptake of cytotoxic agents and patient response. As discussed above, the use of theranostic strategies employing, for example, a TCA and its co-developed diagnostic vector for *in vivo* prescreening of patients for tumor-specific uptake, offers the opportunity to identify patients with the highest likelihood of benefiting from the TCA therapy. Real-time theranostic imaging strategies may offer an alternative or supplemental approach to the more time consuming pharmacogenomics and/or molecular marker signature analyses for predicting response, although these approaches may yet prove complementary rather than mutually exclusive. Moreover, the application of NP or NOP containing vectors that enable their use for therapy in a broader range and higher frequency of cancers may offer better options than antibody-based therapies whose target is likely restricted to a few indications or across several indications at a low frequency. Other targeting agents in addition to NP and NOPs have also been formatted to support theranostic therapies. Studies in several cancers have found that cell surface proteins such as the folate receptor alpha (FOLR1), a highly expressed protein on ovarian and other epithelial derived cancers can be exploited in a theranostic context (53–55). Strategies to develop conjugates

that can be selectively taken up via FOLR1 have been pursued in clinical trials whereby results from these studies have shown that patients whose tumors with uptake of an imaging-folate diagnostic conjugate have enhanced clinical response to a folate–vinblastine therapeutic conjugate compared to patients who do not have folate diagnostic vector uptake (56). Similar approaches to develop conjugate-imaging/conjugate-therapeutic vector pairs have suggested improved patient selection and therapeutic responses. Other examples of diagnostic and therapeutic targeting vector pairs have employed NP technologies to co-develop complexes containing diagnostic agents and an anti-cancer agent, including siRNAs (57). In all cases, the use of TCA and a co-developed targeting diagnostic vector offer alternative methods for delivering personalized therapies to patients in need of new treatments. The key, therefore, for the successful and continued evolution toward personalized medicine is co-development of both the therapeutic and the diagnostic agents as well as diagnostic modalities beginning at the time of target discovery and preclinical studies and continuing through clinical validation and regulatory approvals.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 April 2014; accepted: 24 May 2014; published online: 13 June 2014.
*Citation: Nicolaides NC, O'Shannessy DJ, Albone E and Grasso L (2014) Co-development of diagnostic vectors to support targeted therapies and theranostics: essential tools in personalized cancer therapy. Front. Oncol. **4**:141. doi: 10.3389/fonc.2014.00141*
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology.

frontiers in ONCOLOGY

REVIEW ARTICLE
published: 25 December 2013
doi: 10.3389/fonc.2013.00313

Biomarker-guided repurposing of chemotherapeutic drugs for cancer therapy: a novel strategy in drug development

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Cancer is a leading cause of mortality worldwide and matters are only set to worsen as its incidence continues to rise. Traditional approaches to combat cancer include improved prevention, early diagnosis, optimized surgery, development of novel drugs, and honing regimens of existing anti-cancer drugs. Although discovery and development of novel and effective anti-cancer drugs is a major research area, it is well known that oncology drug development is a lengthy process, extremely costly and with high attrition rates. Furthermore, those drugs that do make it through the drug development mill are often quite expensive, laden with severe side-effects and unfortunately, to date, have only demonstrated minimal increases in overall survival. Therefore, a strong interest has emerged to identify approved non-cancer drugs that possess anti-cancer activity, thus shortcutting the development process. This research strategy is commonly known as drug repurposing or drug repositioning and provides a faster path to the clinics. We have developed and implemented a modification of the standard drug repurposing strategy that we review here; rather than investigating target-promiscuous non-cancer drugs for possible anti-cancer activity, we focus on the discovery of novel cancer indications for already approved chemotherapeutic anti-cancer drugs. Clinical implementation of this strategy is normally commenced at clinical phase II trials and includes pre-treated patients. As the response rates to any non-standard chemotherapeutic drug will be relatively low in such a patient cohort it is a pre-requisite that such testing is based on predictive biomarkers. This review describes our strategy of biomarker-guided repurposing of chemotherapeutic drugs for cancer therapy, taking the repurposing of topoisomerase I (Top1) inhibitors and Top1 as a potential predictive biomarker as case in point.

Keywords: drug repurposing, irinotecan, TOP1, breast cancer, biomarker

INTRODUCTION

Despite the significant improvements in diagnosis and treatment experienced in the past few decades, cancer remains the leading cause of death worldwide, and deaths from cancer are forecasted to reach a staggering 13.2 million deaths by 2030 (1). What's more, these numbers are only set to worsen, as a result of population aging and growth. Assuming that the estimated cancer-specific and sex-specific trends continue, it is expected that the incidence of all-cancer cases will rise from 12.7 million new cases in 2008 to 22.2 million by 2030 (2). Until now this unremitting increase has been offset by significant improvements in prognosis, as a result of earlier diagnosis, advances in surgical therapy, and the use of radiation therapy and adjuvant systemic treatments; as a consequence the survival rates for most cancers have increased significantly in the past few decades. But, unless novel and dramatically improved therapies are introduced, this compensation is unlikely to persist (2, 3). This is particularly crucial for metastatic disease as, for the large majority of cancers, it presents the biggest problem to medical management, being the main cause of death of cancer patients. In recent years our understanding of cancer biology has improved significantly, and resulted in the development of new targeted anti-cancer therapies such as targeting of the EGF-receptor or VEGF. In spite of the initial hope that agents targeting molecular alterations underlying cancer genesis and progression would provide unparalleled therapeutic benefit, reality proved otherwise. Overall, targeted therapies have shown relatively modest clinical benefit, presumably due to intrinsic resistance of tumors to inhibition of signaling intermediates, due mainly to redundancy in signaling pathways in cancer cells (4–8). As a consequence, these novel treatment modalities are not single-agent treatments as they, most often, are combined with conventional cytotoxic drugs. In short, many of the currently available molecular targeted cancer drugs are very costly, provide modest improvements in overall survival, and have significant side-effects.

REPURPOSING OF CANCER DRUGS AS A DRUG DEVELOPMENT STRATEGY

Although there is an acute need for developing new and better anti-cancer drugs, the lengthy time and astronomical high costs associated with cancer drug development, together with high failure rates and limited efficacy of targeted drugs have necessitated alternative approaches to cancer drug discovery (9). Drug repurposing or repositioning is a promising approach to identify suitable drug candidates for treatment of cancer; essentially it entails finding novel therapeutic indications for already approved drugs (10–14). Departing from this drug development strategy, our laboratory developed a simplified variant to identify novel therapeutic indications for chemotherapeutic agents. Our approach differs from the traditional view of drug repurposing in that we do not investigate established target-promiscuous non-cancer drugs for anti-cancer activity (10, 15, 16), but rather try to find new cancer indications for conventional chemotherapeutic agents. Most types of conventional chemotherapy are considered to kill cancer cells not by one single mechanism but by affecting several pivotal pathways/mechanisms with the sum of cellular effects resulting in cancer cell death. Accordingly, the efficacy of any given chemotherapeutic agent may be difficult to foretell using a single molecular predictor. On the other hand, some key molecules have been identified as major targets for chemotherapy drugs. With the above in mind, one could initiate systematic analyses of gene aberrations, mRNA expression, and/or protein determinations of known key target molecules for given chemotherapeutic drugs, specifically in those cancer types that are not conventionally offered these drugs – a knowledge-driven repurposing strategy.

As the response rates to a specific chemotherapeutic drug might be relatively low in an unselected pre-treated patient population, it is a pre-requisite, that the repurposing strategy includes preselection of those patients with a favorable molecular profile in their cancer cells, i.e., those patients with the highest likelihood of obtaining benefit from the treatment. One reasonable assumption would be that one and the same molecule would be both a major target for a chemotherapeutic drug and a predictive biomarker, a hypothesis that is supported by recent evidence. For example, two meta-analyses recently concluded that breast cancer patients with amplification of the topoisomerase 2A (*TOP2A*) gene have more clinical benefit from treatment with topoisomerase II inhibitors than patients with normal *TOP2A* gene number in their cancer cells (17–19). In the present review, we first describe and discuss experiences with topoisomerase I (Top1) measurements in colorectal cancer (CRC). We then turn to a discussion of the repurposing of DNA Top1 inhibitors for treatment of breast cancer.

REPURPOSING OF TOP1 INHIBITORS – IRINOTECAN

Several classes of cytotoxic agents, such as Top1 inhibitors (irinotecan, topotecan), topoisomerase II inhibitors (etoposide), anthracyclines (epirubicin, doxorubicin, mitoxantrone), taxanes (docetaxel, paclitaxel), anti-mitotics (vinorelbine, eribulin), antimetabolites (capecitabine, gemcitabine), or platinum analogs (cisplatin, carboplatin) can be used for the treatment of cancer, be it in the adjuvant, neoadjuvant, or metastatic setting. Each drug class, in addition to a specific therapeutic profile, has its own characteristic toxicity profile. The interplay of these two parameters determines the clinical use of any given drug class, which in many cases is disease specific. As a consequence, in current clinical practice, various drugs are used following evidence-based recommendations for each cancer type; for instance, drugs such as taxanes and anthracyclines are commonly used for standard treatment of breast cancer but not CRC (20). Conversely, camptothecins are used for standard treatment of CRC but not breast cancer (21). These differences in standard clinical use of chemotherapeutic agents essentially reflect the magnitude of clinical benefit attained by the different drugs in clinical trials for each specific disease. One class of anti-cancer drugs of particular interest to us is that of Top1 inhibitors, in particular irinotecan. Irinotecan is a derivative of camptothecin, and it has a unique pharmacological profile, as Top1 is its only target (22), and therefore an obvious candidate for our knowledge-driven repurposing strategy.

Irinotecan is a prodrug, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin (CPT-11), which is converted by carboxylesterases into its active metabolite, 7-ethyl-10-hydroxycamptothecin (SN-38), a potent Top1 inhibitor (23, 24). SN-38 functions by inhibiting the Top1 enzyme, which plays an essential role in alleviating the topological stresses that arise during DNA replication and transcription by nicking, relaxing, and re-ligating the double-stranded DNA structure (22). The current model for anti-cancer activity by irinotecan revolves around the stabilization of (normally) transient DNA-Top1 complexes (termed "cleavage complexes" or Top1cc) by SN-38, thereby inhibiting subsequent re-ligation of the nicked DNA strand. Following the collision of DNA or RNA polymerases into the SN-38-stabilized Top1cc, DNA damage occurs. It has been suggested that upon collision with a DNA polymerase, double-strand breaks are formed, whereas RNA polymerase collision causes the formation of irreversible Top1cc-associated single strand breaks (22, 25, 26). Unless repaired, this DNA damage can lead to cell death [reviewed in (27)].

TOP1 INHIBITORS IN ROUTINE CANCER TREATMENT

Irinotecan and topotecan are the two Top1 inhibitors routinely used in cancer treatment (**Table 1**). In both Europe and the United States (US) irinotecan is recommended by national guidelines as first or second line treatment for metastatic CRC (28, 29). Recently, the combination of 5 FU, irinotecan, and oxaliplatin (FOLFIRINOX) has been recommended in European guidelines on pancreatic cancer, for patients with metastatic disease, $\leq$75 years of age with a good performance status (30). American guidelines, however, do not recommend the use of irinotecan for the treatment of advanced pancreatic cancer (31). A new liposomal formulation of irinotecan (MM-398) has recently been tested in a large phase II study in patients with metastatic pancreatic cancer. Patient recruitment has been completed, however, no results have been published yet (32).

Topotecan is recommended for later line treatment of metastatic ovarian cancer in both Europe and the US (33, 34). Also in both Europe and the US topotecan in combination with cisplatin is approved for the treatment of recurrent cervical cancer (35, 36). Although European guidelines refer an overall survival

Table 1 | Approved and recommended indications for the use of irinotecan and topotecan.

	Europe	United States
IRINOTECAN		
Metastatic colorectal cancer	X	X
Metastatic pancreatic cancer	X	
Metastatic small-cell lung cancer	X	X
TOPOTECAN		
Metastatic ovarian cancer	X	X
Metastatic cervical cancer	X[a]	X
Metastatic small-cell lung cancer	X	

[a]*Approved by authorities but not recommended in clinical guidelines.*

advantage with topotecan in combination with cisplatin compared to monotherapy, combination therapy with topotecan is not recommended for the treatment of metastatic cervical cancer (37). European guidelines on small-cell lung cancer (SCLC) recommend combinations of irinotecan–cisplatin, or topotecan–cisplatin as alternative treatment options for metastatic disease in the case of contraindications to etoposide (a topoisomerase II inhibitor) (38). US guidelines recommend irinotecan combined with cisplatin (among other regimens) as first line treatment for metastatic SCLC and irinotecan monotherapy as second line therapy (39).

A search on clinicaltrials.gov revealed that irinotecan and topotecan alone or in combination with other drugs, currently are being investigated for numerous other indications including various brain tumors, sarcomas, non-small-cell lung cancer (NSCLC), triple negative breast cancer, and gastric, esophageal, and gastroesophageal junction cancers (32). Finally, etirinotecan is a new polymer conjugate of irinotecan (NKTR-102). This drug formulation has a half-life of approximately 50 days compared to 5 days for irinotecan and has shown a lower maximum concentration resulting in greater systemic exposure to SN-38 compared to irinotecan (40). It is currently investigated for the indications SCLC, NSCLC, glioblastomas, and breast cancer (32, 41, 42).

TOPOISOMERASE I GENE STRUCTURE, EXPRESSION, AND ACTIVITY IN CANCER

The topoisomerase I (*TOP1*) gene is located at 20q12, a region that frequently undergoes copy-number alterations across cancer types, including melanoma, breast, colorectal, ovarian, and gastric cancer (43–47). These copy-number alterations have been reported to occur as either gains of chromosome 20, 20q, or as amplification of smaller chromosomal regions, termed "amplicons." Research suggests that in CRC, *TOP1* copy number increases occur predominately in conjunction with the rest of 20q (44–48). Amplification of the *TOP1* gene is observed in a subset of *TOP1* gains, and interestingly, these two types of copy number increases appear to have differential prognostic effects in stage III CRC patients (49). We have recently applied a *TOP1*/CEN-20 fluorescence *in situ* hybridization (FISH) probe mixture to explore the *TOP1* gene copy numbers in stage III CRC (44, 48). The *TOP1* and CEN-20 signals from unaffected epithelial mucosa ($n = 50$) located adjacent to the tumor cells were applied to determine the diploid copy numbers in non-cancer cells. Based on these non-cancer signals we found that 84% of the tumor samples demonstrated an increased *TOP1* gene copy number and 64% had an increased *TOP1*/CEN-20 ratio compared with the non-affected mucosa (44). Of the 50 stage III CRC patients, 13 (26%) had more than 4 *TOP1* copies/cells and 16 (32%) had a *TOP1*/CEN-20 ratio above 1.5 (44). In another study we included 154 stage III CRC chemonaïve patients and found that 55 (35.7%) of the tumors had an increased *TOP1* copy number above 4*n* gene copies per cell and 44 (28.6%) had a *TOP1*/CEN-20 ratio above 1.5 (48). There was no significant correlation between the *TOP1* copy number and proliferation, while multivariate analyses demonstrated a prognostic value since the *TOP1* copy number was significantly associated with overall survival (48). In gastric cancer, several amplicons have been observed on 20q, including one encompassing the *TOP1* gene (43). In malignant melanoma, high level amplifications of the *TOP1* locus can be detected by FISH, indicating the presence of an amplicon, which includes *TOP1* (45). In breast cancer, several amplicons mapped to 20q have been identified, including one covering the 20q12-q13 region (46). By FISH analyses we have established the normal range of *TOP1* copy numbers and found that 31% of primary breast cancer patients have *TOP1* copy number gains ($\geq$4 copies) (50). However, it does not appear that *TOP1* is part of the minimal common region of amplification, indicating that its amplification may occur as a passenger to events involving of an amplicon located at 20q13.1–q13.2 (51). A similar finding has been made in ovarian cancer (47). Taken together, the *TOP1* locus appears to undergo frequent copy number increases in several cancer types. These aberrations are either focal in nature, i.e., amplicon-driven, or may involve larger chromosomal regions, such as 20q. Numerous candidate oncogenes located on 20q have been suggested as the targets of these copy-number alterations. Putative targets include *BCL2L1* (20q11.21), *AIB1* (20q12), and *AURKA* (20q13.2), which have all been implicated in cancer (43, 52, 53). Whether *TOP1* is truly the target of these copy number increases, or whether these increases occur as passenger-related events targeting alternative oncogenes, remains to be elucidated.

Beyond the *TOP1* copy-number alterations at the genomic level, there is also frequent over-expression of *TOP1* mRNA, Top1 protein or enzyme activity level in various cancer types compared to normal adjacent non-cancerous tissue (54–56). Generally, there appears to be a positive correlation between gene expression level, protein level, and activity in cancer tissues (54–56).

COLORECTAL CANCER

Colorectal cancer is the most thoroughly examined cancer with regard to Top1 expression and several studies have found increased Top1 protein in CRC tissues compared to non-cancerous tissues. Already in 1989, immunoblot analyses were applied to show that Top1 protein levels were 14- to 16-fold higher in primary colon adenocarcinoma tissue ($n = 38$) than in normal colonic mucosa (57). Approximately 20–30% of the tumors presented with very high levels of Top1 expression, whereas all normal tissue samples had low levels. Subsequent studies have largely confirmed these data finding 2- to 40-fold increases of *TOP1* mRNA, Top1 protein, or activity (55, 58) in cancer tissue. Copy-number analyses showed that *TOP1* was amplified in 23% of Dukes' C CRC

patients ($n = 52$) when compared to paired normal colon tissue and these *TOP1* amplified tumors had approximately two-times higher RNA level and protein expression level than did the diploid tumors (56). The enzyme activity of Top1 has also been evaluated in crude nuclear extracts from CRC and normal tissue. These data showed that the Top1 activity was significantly higher in primary tumor tissue compared to normal tissue ($n = 53$) (59). In concordance with Giovanella et al. (57), it was found that 20–30% of the tumors possessed very high Top1 activity although the coefficients of variations in these analyses were about 75–80% indicating that these data may be somewhat ambiguous.

Studies have also compared the Top1 protein levels and activity in metastatic CRC tissue to normal tissue and to primary CRC tissue. These data are so far inconclusive. Apparently, the Top1 activity was significantly lower in liver metastases than in the normal liver ($n = 8$) (59). The *TOP1* mRNA levels in FFPE samples did not show significant changes when comparing primary CRC tumor and liver metastasis ($n = 33$) (60) whereas the levels of Top1 protein expression were higher in malignant cells from tumor recurrences compared to primary tumors ($n = 40$) (61) and $n = 25$ (62). Yet another study found concordance between Top1 protein levels in paired primary CRC and lymph node metastases in 33 of 42 cases (63).

Other studies have investigated the protein levels of Top1 in primary tumor CRC tissue by Immunohistochemistry (IHC). These studies have found high Top1 expression in 45% ($n = 62$) of metastatic CRC patients that received a first line 5 FU/CPT-11 chemotherapy (64), 86% ($n = 29$) of primary colon cancers (65), 31% ($n = 13$) among patients with recurrent CRC (66), 17% ($n = 1{,}313$) in metastatic CRC patients (67), and a study comprising 498 Dukes' stage B and C patients reported positive/high Top1 protein expression in 48% of the cases (68). These differences may be due to differences among the studied patient cohorts, choice of antibodies, tissue micro arrays (TMAs) versus full section analyses and scoring systems. In brief, *TOP1* mRNA, Top1 protein, and activity are increased in CRC tissues in comparison to non-cancerous tissues and a substantial subgroup of CRC patients has high levels of Top1.

OTHER CANCERS

Elevated levels of Top1 have also been reported in various other cancers. In poorly differentiated ovarian carcinomas the activity of Top1 was found to be much higher than in non-cancerous tissue or benign tumors (54, 69). In support of this, IHC analyses demonstrated that Top1 protein is primarily associated with tumor cells and much less to normal infiltrating cells (70) and increased Top1 protein expression was found in 43% of ovarian carcinomas (71). Prostate tumors also possessed increased levels of Top1 protein levels and Top1 activity compared to matched non-cancerous tissues, whereas no difference between malignant and normal tissue was found in kidney tumors (55). Similar over-expression of Top1 protein have been reported in urinary bladder carcinomas (77%) (72), gastric carcinomas (68%) (73), testicular tumors (74), renal cell carcinomas (36–100%) (75), malignant melanomas (42%) (76), squamous cell carcinomas (92%) (77), and sarcomas (13%) (78). In metastatic breast cancer (mBC) the Top1 protein expression has been evaluated by IHC in FFPE tissue from 22 primary breast cancer. It was found that 41% over-expressed Top1 (79). Interestingly, the expression of Top1 protein varies from undetectable to strongly positive among the analyzed samples, which indicate that Top1 expression may be a suitable biomarker in a subgroup of mBC patients.

CLINICAL STUDIES EVALUATING THE PREDICTIVE ROLE OF TOP1

Until now the association between Top1 assessed in tumor tissue and irinotecan efficacy has only been investigated retrospectively and with focus on CRC. Top1 levels have been determined by IHC where protein expression was assessed (64, 67, 68) and by RT-PCR where gene expression (mRNA) was analyzed (60). Tumor samples were obtained from patients who were originally enrolled in randomized phase III trials or from patients routinely treated in accordance with current local clinical guidelines.

Two small single-cohort biomarker studies investigated patients with advanced CRC who were all treated with different regimens of 5 FU/leucovorin + irinotecan (60, 64, 67, 68). These studies did not identify any significant association between *TOP1* gene expression or Top1 protein expression and objective response rates or survival endpoints. However, both studies were methodologically flawed as Top1 data was only available from 62 to 33 patients, respectively, and due to the consequent inherent lack of sufficient statistical power, this makes it almost impossible to obtain statistically significant results even though the association in question was in fact true. Additionally, a true distinction between a predictive and a prognostic component of a biomarker will not be identified when survival analysis is performed in a single-cohort study without a relevant control group (80, 81).

Biomarker studies designed to obtain Level of Evidence (LoE) 1 as proposed by Simon et al. (81) have been conducted where material from randomized clinical phase III trials was used in order to conduct a so-called prospective-retrospective biomarker evaluation according to a stringent analysis plan. In a study by Braun et al. (67) primary tumor material from patients originally accrued in the UK MRC FOCUS study (82) was used. The UK MRC FOCUS study was a randomized clinical trial investigating different combinations of chemotherapy for patients with advanced CRC. In first line 1,628 patients were randomized between 5 FU/levofolinate, 5 FU/levofolinate + irinotecan or 5 FU/levofolinate + oxaliplatin. As patients in the 5 FU/levofolinate arm could be used as relevant controls to correct for potential concurrent prognostic qualities of the biomarkers in question, this clinical design was ideal when investigating putative predictive biomarkers of either irinotecan or oxaliplatin efficacy. In the biomarker study, Top1 protein expression was assessed by IHC using a murine monoclonal antibody (clone 1D6, Novocastra), and the staining intensity was graded as low, moderate, or high. Due to inadequate tumor material or failed IHC analysis 315 cases were excluded, which resulted in available Top1 data from 1,313 tumor samples (81%). The authors reported a significant association between staining intensity and progression free survival where patients with tumors showing moderate or high expression benefited from the addition of irinotecan compared to 5 FU/levofolinate therapy alone. In contrast, patients with Top1 low classified tumors did not benefit more from the irinotecan combination than from the 5 FU/levofolinate treatment alone.

The interaction between Top1 and the irinotecan combination was reported to be statistically significant ($P = 0.001$). An attempt to validate these results was performed in tumor material from the CAIRO trial (83). In the CAIRO trial, 820 patients with advanced CRC were originally randomized between capecitabine, a prodrug of 5 FU, or capecitabine + irinotecan as first line treatment. In the following biomarker study (84), which was only published in abstract form, tumor samples from 545 patients were included and the same methodologies as in the study by Braun et al. (67) were applied. The study failed to confirm the positive association between Top1 protein expression and irinotecan efficacy. There are several explanations to why confirmation failed. First, the hypothesis may not be correct, and second, potential methodological bias may have been introduced unintentionally. Assessment of IHC staining intensity can be problematic and inter-observer variability due to staining heterogeneity and the somewhat subjective nature of the evaluation is a challenge to this methodology. Additionally, information on analytical validation of the applied antibody is essential to ensure prober sensitivity and specificity, and to our knowledge this is lacking for the 1D6 clone, which was used in both the CAIRO and the UK MRC FOCUS trials – as a result we cannot objectively determine which trial, if any, may be at fault. However, as stated previously, both the CAIRO and the UK MRC FOCUS trials fulfill the requirements set by Simon et al. (81) in order to obtain LoE 1 for a predictive biomarker of irinotecan efficacy, and the trials still represent the best available option to retrospectively assess the association between other biomarkers or Top1 analyzed by techniques other than IHC and irinotecan in the advanced setting of CRC.

The association between Top1 protein expression and irinotecan efficacy has also been investigated in the adjuvant setting of CRC, and results from a retrospective biomarker study suggested a positive predictive role of Top1 protein expression (68). The study did not use material from one randomized clinical trial but included material from several clinical trials, which resulted in two cohorts of patients who were either treated with 5 FU/leucovorin alone or 5 FU/leucovorin + irinotecan. However, this methodology was intrinsically flawed as the original clinical trials spanned almost two decades, a time frame in where great surgical improvements in the managements of CRC have taken place.

Based on negative results from several phase III trials, i.e., the PETACC-3 (85) and the CALGB 89803 (86), the 5 FU/leucovorin + irinotecan combination is today not recommended in the adjuvant setting of colon cancer. However, as patients in these trials were randomized between 5 FU/leucovorin and 5 FU/leucovorin + irinotecan, tumor tissue from these trials is highly appropriate for retrospective biomarker research in relation to prediction of irinotecan efficacy. The main challenge with such an approach is the availability of a sufficient number of tumor samples to obtain the necessary statistical power.

DESIGN OF CLINICAL STUDIES TO VALIDATE PREDICTIVE BIOMARKERS

Repurposing often involves drugs where the mechanisms of action are fully or partly known. Thus, clinical repurposing trials may take the advantage of such knowledge and from early phase development/testing include predictive biomarkers. Such biomarkers will often be found among molecules known to be mechanistically involved in sensitivity/resistance to the drug. The use of predictive biomarkers in early drug testing may increase the therapeutic index of the drug in question by increasing the efficacy of the drug in the selected biomarker favorable population and at the same time avoid drug-induced toxicity in the biomarker unfavorable population as these patients will not be exposed to the drug. Looking ahead, future drug indications might be limited to small subgroups of patients based on predictive biomarkers. Targeted drug selection is already in routine use e.g., estrogen receptor and human epidermal growth factor receptor (HER) 2 in breast cancer, *KRAS* in CRC and *BRAF* in malignant melanomas. The status of the relevant marker is frequently based only on analysis of the primary tumor. However, in e.g., breast cancer accumulating evidence suggests that tumor characteristics, including ER and HER2 might change through tumor progression (87). Thus, the treatment strategy may require readiness to perform serial biopsies, including biopsies from metastatic lesions. The statistical considerations and principles for repurposing an old drug accompanied by a biomarker are exactly the same as for the development of a new (targeted) drug.

PHASE I

Most often, repurposing of an old drug will not involve a phase I trial. However, new knowledge concerning relevant biomarkers might encourage the clinicians to try new drug combinations and thus perform a phase I trial.

The goal of incorporating biomarkers in this stage of development is a better characterization of the biomarker and the assay performance in human samples (88). In this context the present EMA guidelines urge investigators of non-cytotoxic products to analyze not only biopsies from the primary tumor and metastasis but also normal tissue to understand the molecular background for efficacy (89). More recently, molecular pre-screening has been suggested for selecting patients for early drug development. Thus, it is envisioned that academic institutions establish molecular pre-screening programs in order to select patients for phase I trials (90).

PHASE II

The biomarker should be included for hypothesis testing and early indications for proof-of concept. There are two types of clinical trial designs effective in evaluating the role of a potential predictive biomarker in phase II: the adaptive parallel two stage design and the tandem two-step predictor biomarker evaluation trial design. The designs and rational behind them have been reviewed by McShane et al. (88).

PHASE III

Phase III studies designed to repurpose an old drug will most often involve late stage cancer patients in order to compare monotherapy with a test drug versus best supportive care. Alternatively, the test drug might be evaluated as an add-on to a known treatment.

Prospectively designed clinical trials are regarded as the gold standard for evaluating a predictive biomarker. In many instances, however, due to time and expenses required for these trials, a retrospective testing of predictive biomarkers is more feasible.

Retrospective validation of biomarkers is regarded as an acceptable strategy in selected circumstances. The strategy requires data from well-designed prospective phase III, randomized trials, sample availability from on a large majority of patients to avoid bias due to patient selection, a prospectively stated hypothesis, a predefined and standardized assay, and upfront sample size and power justification (91). Optimally, evidence should be provided from two independent randomized trials. *KRAS* as a predictor for efficacy of cetuximab and panitumumab in CRC is an example of a biomarker which has successfully been validated using a retrospective strategy.

In general there are four types of clinical trial designs to evaluate a potentially predictive biomarker: (1) the all-comers design with a "biomarker end point" as second objective, (2) a targeted design that restricts the study population to patients who have a favorable predictive biomarker profile, (3) a strategy design which randomizes patients to receive biomarker-based or non-biomarker-based (standard) treatment, and (4) a multiple hypothesis design, which combines the targeted design and the all-comers design. The latter design addresses the multiple hypotheses by having co-primary objectives (91, 92). Each of the designs has potential advantages and disadvantages. The all-comers design requires validation in a separate trial while the other designs prospectively evaluate the biomarker. Choice of design should depend upon knowledge on the biomarker and disease setting (91–94).

The REporting recommendations for tumor MARKer prognostic studies (REMARK) guidelines were developed in order to standardize and improve the quality of cancer biomarker studies. Reporting of results should follow these guidelines (95). More recently, guidelines for conducting experiments using tissue microarrays have been published (96). This checklist should be used in addition to the REMARK guidelines. With a more rational drug development including biomarker driven trials, researchers might ultimately yield greater benefits for patients.

REPURPOSING IRINOTECAN TO BREAST CANCER

Breast cancer is the most common kind of cancer among women. Improved adjuvant treatment in early breast cancer has resulted in better prognosis, but still approximately 20% of women, initially diagnosed with regional disease will develop systemic recurrence within 5 years.

Two major, still unresolved, medical problems are that almost all patients with mBC who obtain an objective response to chemotherapy will eventually experience disease recurrence and death from their disease. Secondly, a large fraction of the patients with mBC who receive first line systemic chemotherapy will not gain any beneficial effects from the treatment. In contrast, they may suffer from drug-induced side-effects and in addition, initiation of a potential effective second line treatment may be delayed until lack of response to the first line treatment is evidenced.

In current treatment of mBC, the main first line cytotoxic drugs are anthracyclines, and/or taxanes combined with cyclophosphamide. Second line treatment may include 5 FU, gemcitabine, platin derivatives, or vinorelbine. Unfortunately, very few options are available as third line treatment. It is thus clear that there is an urgent need for new and effective drugs in this setting. On the other hand, such drugs should be used with caution as they may be associated with significant side-effects with severe influence on the quality of life of the patients. If possible, such drugs should be used in combination with predictive biomarkers, allowing for a personalized treatment approach in which only patients with a high likelihood of an objective response should be offered the treatment in question. A number of publications have demonstrated some benefit from irinotecan treatment in patients with mBC being refractory to current breast cancer treatment (21). However, with a relatively small group of patients obtaining benefit from the treatment and the rather serious side-effects associated with irinotecan treatment, there will be a need for a predictive biomarker profile when introducing irinotecan in the treatment of mBC. We describe here, using the example of repurposing of Top1 inhibitors for the treatment of breast cancer, our approach to identify novel therapeutic indications for standard chemotherapeutic agents, based on prior knowledge of the pharmacology of these agents and exploratory studies for biomarker establishment.

The gene expression level of *TOP1* may not always predict response to camptothecin (97, 98) and the currently available antibodies to the Top1 protein have not yet been sufficiently validated. FISH is a validated clinical method to be used on FFPE tissue and it provides a direct measure of cancer cell gene aberrations on a cell to cell basis and may therefore provide more specific information than global genomics techniques. Therefore, we have used a *TOP1*/CEN-20 FISH probe mix to determine the *TOP1* gene aberration frequency in clinical breast cancer biopsies ($n = 100$) and compared to findings in normal breast tissue ($n = 100$). These data demonstrated that *TOP1* gene copy numbers of normal breast tissues were all in the diploid range, whereas 31% of the breast cancer samples had *TOP1* copy number gain ($\geq$4 copies) (50). In breast cancer tissue we have observed a significant association between the *TOP1* copy numbers and the *TOP1* mRNA expression (50) which in combination with the frequent amplification of the *TOP1* gene suggest that *TOP1* gene copy numbers may be clinically relevant as a potential predictive biomarker for irinotecan sensitivity in breast cancer. Based on our FISH data and published reports on the response rates of irinotecan in mBC (21), we have initiated two clinical phase II trials with mBC patients being refractory to anthracyclines and taxanes. Patients with *TOP1* copy number gain ($\geq$4 copies) are offered treatment with irinotecan. The patients are stratified according to HER2 levels being either HER2-positive (POSIRI; EudraCT 2012-002347-23) or HER2-negative (NEGIRI; EudraCT and 2012-002348-26). The main goal is to get objective response rate according to RECIST 1.1. In **Figure 1** we have exemplified our approach to biomarker-guided repurposing of irinotecan in breast cancer by picturing two individual ER-positive and HER2-neutral breast cancer patients. These patients possess either *TOP1* copy numbers in the normal range (**Figure 1A**) or increased *TOP1* copy numbers (**Figure 1B**) and only the latter would therefore be eligible for irinotecan therapy. If these studies and a subsequent phase III trial are positive, the *TOP1* copy number may be applied as a predictive biomarker for irinotecan treatment in anthracycline and/or taxane refractory mBC. Additionally, an association between *TOP1* copy numbers and irinotecan effect should subsequently be tested in the other cancer types not currently being treated with irinotecan. We believe that the workflow described here can be applied to

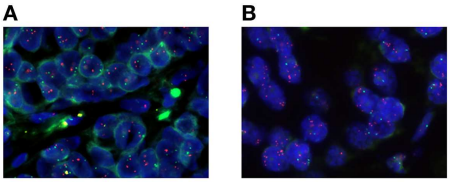

FIGURE 1 | Microscope photographs of two different primary breast cancer specimens stained with a fluorescent *TOP1*/CEN-20 fluorescence *in situ* hybridization (FISH) probe mix. Red spots visualize the *TOP1* gene and green spots represent CEN-20. **(A)** A breast cancer specimen with diploid *TOP1* copy number of 2.97, CEN-20 copy number of 1.90, and a ratio of 1.56. **(B)** A breast cancer specimen with amplified *TOP1* copy number of 6.35, CEN-20 copy number of 1.90, and a ratio of 3.34.

other chemotherapeutic drugs and/or other indications, providing a viable shortcut to novel effective treatments.

CONCLUSION

Currently, few people would argue against that the future of drug development in oncology lies with the identification of predictive biomarkers capable of identifying those subsets of patients who will benefit from a given therapy. The use of biomarkers to pinpoint those with a favorable response profile, normally a small subgroup of patients, within a large population is at the heart of the concept of personalized medicine. Also, the use of companion molecular diagnostics promise to minimize the size, costs, and failure rates of cancer agents in clinical trials.

We describe here our strategy of biomarker-guided repurposing of chemotherapeutic drugs for cancer therapy, exemplified with the repurposing of Top1 inhibitors and Top1 as a potential predictive biomarker. This approach can conceivably be implemented to a substantial number of currently used chemotherapeutic drugs, since their mechanisms of action are well studied with thousands of studies available in the literature. We believe that this strategy is valuable and can, potentially, add new tools to the armamentarium of drugs at the disposal of oncologists.

ACKNOWLEDGMENTS

DAKO A/S, Denmark for providing the *TOP1* probe mix. This work was supported by Danish National Research Foundation: Sino-Danish Breast Cancer Research Centre; A Race Against Breast Cancer; Danish Center for Translational Breast Cancer Research; the IMK Almene Fond and the Danish Cancer Society.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 October 2013; paper pending published: 06 November 2013; accepted: 10 December 2013; published online: 25 December 2013.
*Citation: Stenvang J, Kümler I, Nygård SB, Smith DH, Nielsen D, Brünner N and Moreira JMA (2013) Biomarker-guided repurposing of chemotherapeutic drugs for cancer therapy: a novel strategy in drug development. Front. Oncol. **3**:313. doi: 10.3389/fonc.2013.00313*
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology.

frontiers in
ONCOLOGY

REVIEW ARTICLE
published: 23 December 2013
doi: 10.3389/fonc.2013.00315

Drug-diagnostics co-development in oncology

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Developments in genomics are providing a biological basis for the heterogeneity of clinical course and response to treatment that have long been apparent to clinicians. The ability to molecularly characterize human diseases presents new opportunities to develop more effective treatments and new challenges for the design and analysis of clinical trials. In oncology, treatment of broad populations with regimens that benefit a minority of patients is less economically sustainable with expensive molecularly targeted therapeutics. The established molecular heterogeneity of human diseases requires the development of new paradigms for the design and analysis of randomized clinical trials as a reliable basis for predictive medicine. We review prospective designs for the development of new therapeutics and predictive biomarkers to inform their use. We cover designs for a wide range of settings. At one extreme is the development of a new drug with a single candidate biomarker and strong biological evidence that marker negative patients are unlikely to benefit from the new drug. At the other extreme are phase III clinical trials involving both genome-wide discovery of a predictive classifier and internal validation of that classifier. We have outlined a prediction based approach to the analysis of randomized clinical trials that both preserves the type I error and provides a reliable internally validated basis for predicting which patients are most likely or unlikely to benefit from a new regimen.

Keywords: predictive biomarker, clinical trial design, adaptive design, companion diagnostic, enrichment trial

INTRODUCTION

This dominant paradigm for oncology drug development has been rapidly changing. The paradigm for development of cytotoxics involved large phase III clinical trials to find relatively small, but statistically significant, average treatment effects for target populations defined in terms of primary site and stage. The primary analysis was relatively simple, consisting of a single statistical test of the null hypothesis of no average treatment effect for the intent to treat population with regard to a single primary endpoint. Any claim of treatment benefit based on subset analysis without an overall statistically significant intent to treat analysis was viewed with suspicion.

Randomized clinical trials have made important contributions to modern medicine and public health, but they have also led to the over-treatment of broad populations of patients, most of whom don't benefit from the increasingly expensive drugs and procedures shown to have statistically significant average treatment effects in increasingly large clinical trials. With the recognition of the molecular heterogeneity of cancer and the development of molecularly targeted drugs whose effects depend strongly on the genomic alterations and genetic background of the tumor, the broad eligibility primary site oriented clinical trial is playing a less dominant role. Increasingly sophisticated and cost effective biotechnology platforms are providing the tools to develop diagnostics that identify the patients most likely to benefit from molecularly targeted drugs.

Tumors of a primary site in many represent a heterogeneous collection of diseases that differ in pathophysiology, natural history, and sensitivity to treatment. These diseases differ with regard to the mutations that cause them and drive their invasion. The heterogeneous nature of tumors of the same primary site offers new challenges for drug development and clinical trial design. Physicians have always known that cancers of the same primary site were heterogeneous with regard to natural history and response to treatment. This understanding sometimes led to conflicts with statisticians over the use of subset analysis in the analysis of clinical trials. Although most statisticians expressed concern about the potential for false positive findings results from *post hoc* subset analysis, some practitioners rejected the results of clinical trials whose conclusions were based on average effects. Today we have better tools for characterizing the tumors biologically and using this characterization in the design and analysis of clinical trials that utilize this information prospectively.

Most oncology drugs are being developed for defined molecular targets. In some cases the targets are well understood and there is a compelling biological basis for restricting development to the subset of patients whose tumors are characterized by deregulation of the drug target. For other drugs there are multiple targets and more uncertainty about how to measure whether a drug target is driving tumor invasion in an individual patient (1). It is clear that the primary analysis of the new generation of oncology clinical trials must consist of more than just treating broad patient populations and testing the null hypothesis of no average effect. But it is also clear that the tradition of *post hoc* data dredging subset analysis is not an adequate basis for predictive oncology. For establishing practice standards and for drug approvals we need prospective analysis plans that provide for both preservation of the type I experiment-wise error rate and for focused predictive

analyses that can be used to reliably select patients in clinical practice for use of the new regimen (2–4). These two primary objectives involve co-development of a drug and a companion diagnostic.

In the following sections we summarize some of the designs that are available for the co-development of a drug and companion diagnostic. Developing new treatments with companion diagnostics or predictive biomarkers for identifying the patients who benefit does not make drug development simpler, quicker, or cheaper as is sometimes claimed. Actually it makes drug development more complex and probably more expensive. But for many new oncology drugs it should increase the chance of success. It may also lead to more consistency in results among trials and increase the proportion of patients who benefit from the drugs they receive. This approach also has great potential value for controlling societal expenditures on health care.

The ideal approach to co-development of a drug and companion diagnostic involves: (i) identification of a predictive biomarker based on understanding the mechanism of action of the drug and the role of the drug target in the pathophysiology of the disease. This biological understanding should be validated and refined by pre-clinical studies and early phase clinical trials. The predictive biomarkers for successful cancer drugs have generally involved a single gene or protein rather than a multivariate classifier. Multivariate classifiers have been found some use as prognostic indicators that reflect a combination of the pace of the disease and the effect of standard therapy. Multivariate classifiers have rarely been used as predictive biomarkers for response to specific drugs because their use often reflects an incomplete understanding of the mechanism of action of the drug or the role of its molecular target. (ii) Development of an analytically validated test for measurement of that biomarker. Analytically validated means that the test accurately measures what it is supposed to measure, or if there is no gold-standard measurement, that the test is reproducible and robust. (iii) Use of that test to design and analyze a new clinical trial to evaluate the effectiveness of that drug and how the effectiveness relates to the biomarker value.

In the enrichment and stratified designs described below, biomarker discovery and determination of the threshold of positivity is performed prior to the phase III trial. Cancer biology is complex, however, and it is not always possible to have everything sorted out in this way before launching the phase III clinical trials. We will also discuss designs and prospective analysis plans that permit one to adaptively determine the best threshold of positivity for the biomarker and designs that incorporate multiple candidate biomarkers.

TARGETED (ENRICHMENT) DESIGNS

Designs in which eligibility is restricted to those patients considered most likely to benefit from the experimental drug are called "targeted designs" or "enrichment designs." With an enrichment design, the analytically validated diagnostic test is used to restrict eligibility for a randomized clinical trial comparing a regimen containing a new drug to a control regimen. This approach has now been used for pivotal trials of many drugs whose molecular targets were well understood in the context of the disease. Several authors (5–9) studied the efficiency of this approach relative to the standard approach of randomizing all patients without using the biomarker test at all. The efficiency of the enrichment design depends on the prevalence of test positive patients and on the effectiveness of the new treatment in test negative patients. When fewer than half of the patients are test positive and the new treatment is relatively ineffective in test negative patients, the number of randomized patients required for an enrichment design is dramatically smaller than the number of randomized patients required for a standard design. For example, if the treatment is completely ineffective in test negative patients, then the ratio of number of patients required for randomization in the enrichment design relative to the number required for the standard design is approximately $1/\gamma^2$ where γ denotes the proportion of patients who are test positive. The treatment may have some effectiveness for test negative patients either because the assay is imperfect for measuring deregulation of the putative molecular target or because the drug has off-target anti-tumor effects. Even if the new treatment is half as effective in test negative patients as in test positive patients, however, the randomization ratio is approximately $4/(\gamma+1)^2$. This equals about 2.56 when $\gamma = 0.25$, i.e., 25% of the patients are test positive, indicating that the enrichment design reduces the number of required patients to randomize by a factor of 2.56.

The enrichment design was very effective for the development of trastuzumab even though the test was imperfect and has subsequently been improved. Simon and Maitournam (5, 6) also compared the enrichment design to the standard design with regard to the number of screened patients. We have made the methods of sample size planning for the design of enrichment trials available on line at http://brb.nci.nih.gov. The web-based programs are available for binary and survival/disease-free survival endpoints. The planning takes into account the performance characteristics of the tests and specificity of the treatment effects. The programs provide comparisons to standard non-enrichment designs based on the number of randomized patients required and the number of patients needed for screening to obtain the required number of randomized patients.

The enrichment design is appropriate for contexts where there is a strong biological basis for believing that test negative patients will not benefit from the new drug. In such cases, including test negative patients may raise ethical concerns and may confuse the interpretation of the clinical trial. As described in the section on "stratification designs," if test negative patients are to be included then one should ensure that a sufficient number of test positive patients are included to provide an adequately powered evaluation. Often this is not done and instead one sees a mixed population of patients in an inadequately sized trial leading to ambiguous conclusions.

The enrichment design does not provide data on the effectiveness of the new treatment compared to control for test negative patients. Consequently, unless there is compelling biological or phase II data that the new drug is not effective in test negative patients, the enrichment design may not be adequate to support approval of the test. If the biological rationale or phase II data is strong, however, then the test can be approved for identifying a subset of patients for whom an effective drug exists, rather than for distinguishing patients who do and do not benefit from the new drug.

In oncology, sequencing of tumor DNA to test for point or structural alterations in genes whose protein products are druggable is rapidly becoming part of the standard diagnostic workup at advanced cancer centers. Regulatory body approvals of drugs for populations defined by such tests will require that the tests be shown to have good analytical performance (10).

BIOMARKER STRATIFIED DESIGN

When a predictive classifier has been developed but there is not compelling biological or phase II data that test negative patients do not benefit from the new treatment, it is generally best to include both classifier positive and classifier negative in the phase III clinical trials comparing the new treatment to the control regimen. In this case it is essential that an analysis plan be pre-defined in the protocol for how the predictive classifier will be used in the analysis. The analysis plan will generally define the testing strategy for evaluating the new treatment in the test positive patients, the test negative patients, and overall. The testing strategy must preserve the overall type I error of the trial and the trial must be sized to provide adequate statistical power for these tests. It is not sufficient to just stratify, i.e., balance, the randomization with regard to the classifier without specifying a complete analysis plan. The main value of "stratifying" (i.e., balancing) the randomization is that it assures that only patients with adequate test results will enter the trial. Pre-stratification of the randomization is not necessary for the validity of inferences to be made about treatment effects within the test positive or test negative subsets. If an analytically validated test is not available at the start of the trial but will be available by the time of analysis, then it may be preferable not to pre-stratify the randomization process.

The purpose of the pivotal trial is to evaluate the new treatment overall and in the subsets determined by the pre-specified classifier (generally biomarker plus cut-point for positivity). The purpose is not to modify or optimize the classifier unless an adaptive design is used. Several primary analysis plans have been described (10–12) and a web-based tool for sample size planning for some of these analysis plans is available at http://brb.nci.nih.gov For example, If one has moderate strength evidence that the treatment, if effective at all, is likely to be more effective in the test positive cases, one might first compare treatment versus control in test positive patients using a threshold of significance of 5%. Only if the treatment versus control comparison is significant at the 5% level in test positive patients, will the new treatment be compared to the control among test negative patients, again using a threshold of statistical significance of 5%. This sequential approach controls the overall type I error at 5%. To have 90% power in the test positive patients for detecting a 50% reduction in hazard for the new treatment versus control at a two-sided 5% significance level requires about 88 events of test positive patients. If at the time of analysis the event rates in the test positive and test negative strata are about equal, then when there are 88 events in the test positive patients, there will be about $88(1-\gamma)/\gamma$ events in the test negative patients where γ denotes the proportion of test positive patients. If 25% of the patients are test positive, then there will be approximately 264 events in test negative patients. This will provide approximately 90% power for detecting a 33% reduction in hazard at a two-sided significance level of 5%. In this case, the trial will not be delayed compared to the enrichment design, but a large number of test negative patients will be randomized, treated, and followed on the study rather than excluded as for the enrichment design. This will be problematic if one does not, *a priori*, expect the new treatment to be effective for test negative patients. In this case it will be important to establish an interim monitoring plan to terminate accrual of test negative patients when interim results and prior evidence of lack of effectiveness makes it no longer viable to enter them.

In the situation where one has more limited confidence in the predictive marker it can be effectively used for a "fall-back" analysis. In Simon and Wang (13), we proposed an analysis plan in which the new treatment group is first compared to the control group overall. If that difference is not significant at a reduced significance level such as 0.03, then the new treatment is compared to the control group just for test positive patients. The latter comparison uses a threshold of significance of 0.02, or whatever portion of the traditional 0.05 not used by the initial test. If the trial is planned for having 90% power for detecting a uniform 33% reduction in overall hazard using a two-sided significance level of 0.03, then the overall analysis will take place when there are 297 events. If the test is positive in 25% of patients and the event rates in test positive and test negative patients are about equal at the time of analysis, then when there are 297 overall events there will be approximately 75 events among the test positive patients. If the overall test of treatment effect is not significant, then the subset test will have power 0.75 for detecting a 50% reduction in hazard at a two-sided 0.02 significance level. By delaying the treatment evaluation in the test positive patients power 0.80 can be achieved when there are 84 events and power 0.90 can be achieved when there are 109 events in the test positive subset. Wang et al. have shown that the power of this approach can be improved by taking into account the correlation between the overall significance test and the significance test comparing treatment groups in the subset of test positive patients (14). So if, for example a significance threshold of 0.03 has been used for the overall test, the significance threshold for used for the subset can be somewhat >0.02 and still have the overall chance of a false positive claim of any type limited to 5%. Real world experience with stratification and enrichment designs are described by Freidlin et al. (15) and by Mandreakar and Sargent (16). Freidlin et al. (17) describe a randomized phase II design for providing information for the design of the phase III trial in cases where there is not a strong biological rationale for the enrichment approach.

INTERIM MONITORING OF TEST NEGATIVE PATIENTS

Interim monitoring of outcome for the test negative patients is very important in clinical trials where there is preliminary evidence that efficacy of the new regimen may be limited to the test positive patients. One approach is to perform an interim analysis focused on the test negative patients using a standard futility monitoring statistical plan for the primary endpoint of the clinical trial. Such methods are usually either based on the standardized treatment effect or the conditional power of rejecting the null hypothesis at the end of the trial. One simple approach is to compute the standardized treatment effect in the test negative patients at a time when half of the events in test negative patients projected

to occur by the end of the trial have occurred. If the treatment effect is going in the wrong direction, then accrual to the test negative stratum ceases. This type of futility analysis is designed to be conservative enough that the power at the end of the trial for detecting a treatment effect is minimally reduced. This type of futility monitoring is used in the design proposed by Wang et al. (14) but in many cases it provides very limited protection for test negative patients for use in biomarker driven designs. Depending on the accrual rate and survival distributions, by the time half of the primary endpoint events have occurred for the test negative patients, the accrual of test negative patients may be close to complete.

An alternative approach would be to base the futility monitoring of the test negative patients on an intermediate endpoint rather than on the primary endpoint of the trial. There would be no assumption that the intermediate endpoint is a true surrogate for the primary endpoint, only that if there is no treatment effect on the intermediate endpoint, then there is unlikely to be a treatment effect for the primary endpoint. With this limited assumption, made for most phase II trials, the futility analysis can be performed at an earlier time so that a finding of futility will limit the number of test negative patients accrued.

In Karuri and Simon (18) we introduced a phase III design for this setting in which futility monitoring of the test negative patients is performed based on a joint prior joint distribution for the treatment effects in test negative and test positive patients. That prior distribution enables the trial investigator to represent the prior evidence that treatment effect will be reduced for test negative patients and use that information in monitoring the clinical trial. Although the formulation is Bayesian, the rejection region based on posterior probability is calibrated so that type I errors satisfy the usual frequentist requirements.

BIOMARKER ADAPTIVE THRESHOLD DESIGN

In Jiang et al. (19) we reported on a "Biomarker Adaptive Threshold Design" for situations where a biomarker is available at the start of the trial, but a cut-point for converting the value to a binary classifier is not established. For example, this design could be used with a FISH assay for EGFR positivity without pre-specification of the threshold of positivity. Tumor specimens are collected from all patients at entry, but the value of the biomarker is not used as an eligibility criteria. Their analysis plan does not stipulate that the assay for measuring the index needs to be performed in real time. Two analysis plans were described. Analysis plan A begins with comparing outcomes for all patients receiving the new treatment to those for all control patients. If this difference in outcomes is significant at a pre-specified reduced significance level α_1 (e.g., 0.03) then the new treatment is considered effective for the eligible population as a whole. Otherwise, a second stage test is performed using significance threshold $\alpha_2 = 0.05 - \alpha_1$. The second stage test involves finding the cut-point s^* for the biomarker score which leads to the largest treatment effect in comparing T to C restricted to patients with score greater than s^*. Jiang et al. employed a log-likelihood measure of treatment effect and let L^* denote the log-likelihood of treatment effect when restricted to patients with biomarker level above s^*. The null distribution of L^* was determined by repeating the analysis after permuting the treatment and control labels a thousand or more times, recomputing s^* and L^* each time. If the permutation statistical significance of L^* is $<0.05 - \alpha_1$ (e.g., 0.02), then treatment T is considered superior to C for the subset of the patients with biomarker level above s^*.

The advantage of procedure A is its simplicity and that it explicitly separates the test of treatment effect in the broad population from the subset selection. However, the procedure takes a conservative approach in adjusting for multiplicity of combining the overall and subset tests. An alternative analysis plan B proposed by Jiang et al. does not use a first stage comparison of treatment groups overall. Consequently, plan B is more appropriate to settings in which there is greater expectation that treatment effect will be limited to a marker defined subset. With analysis plan B they determine the cut-point value b at which $w(b)S(b)$ is maximized, where $w(b)$ is a pre-defined weight function. The weight function is used to give greater emphasis to the $b = 0$ subset, that is, the subset containing all patients (marker value is initially normalized to the 0–1 interval). Let $T(b) = w(b)S(b)$ denote the value of the maximized weighted partial log-likelihood. The statistical significance of $T(b)$ is determined by generating the null distribution by repeating the optimization procedure for many cases of randomly permuted data. With either procedure A or B, a confidence interval for the optimal cut-point b is generated by bootstrap re-sampling of the maximum likelihood estimate of the cut-point based on a proportional hazards model with an unknown cut-point and an unknown treatment effect for patients with biomarker values above the cut-point. Since the treatment is presumed effective only for patients with biomarker above the threshold b, the confidence coefficient associated with a given biomarker value x can be interpreted as the probability that a patient with marker value x benefits from the new treatment.

In Jiang et al. (19) we also provided an approach to sample size planning for the biomarker adaptive threshold design. With analysis strategy A, sample size is determined in the traditional manner for overall comparison of the treatment arms but powering the trial for using a reduced significance level a_1, e.g., 0.03. With analysis plan B a larger sample size is used to provides good power for establishing the statistical significance of treatment effects restricted to patients with biomarker values above an initially unknown cut-point.

ADAPTIVE ENRICHMENT DESIGNS

The adaptive threshold design described above (19) enables one to conduct the phase III clinical trial without pre-specifying the cut-point for the biomarker. It provides for a valid statistical significance test that has good statistical power against alternative hypotheses that the treatment effect is limited to patients with biomarker values above some unknown level, and it provides a confidence interval for estimation of the cut-point. These analyses are, however, performed at the end of the trial and accrual during the trial is not restricted by biomarker value. In Simon and Simon (20), we introduced a very general class of adaptive enrichment designs in which the eligibility criteria are adaptively adjusted during the course of the trial in order to exclude patient subsets unlikely to benefit from the new regimen. Others have also studied adaptive enrichment designs (21–23). Wang et al. (21) and Simon and Simon (20) provide general frameworks for adaption

and identify statistical significance tests that provide protection of the study-wise type I error under broad conditions. In Simon and Simon (20) we applied this framework to the setting of adaptive threshold enrichment of a single biomarker.

DESIGNS THAT EVALUATE A SMALL NUMBER OF BIOMARKERS

Because of the complexity of cancer biology, there are many cases in which the biology of the target is not sufficiently well understood at the time that the phase III trials are initiated to restrict attention to a single predictive biomarker. The analysis plan used in the adaptive threshold design (19) is based on computing a global test based on a maximum test statistic. For the adaptive threshold design, the maximum is taken over the set of cut-points of a biomarker score. The idea of using a global maximum test statistic is much more broadly applicable, however. For example, suppose multiple candidate binary tests, $B_1, \ldots, B_K$ are available at the start of the trial. These tests may or may not be correlated with each other. Let L_k denote the log-likelihood of treatment effect for comparing T to C when restricted to patients positive for biomarker k. Let L^* denote the largest of these values and let k^* denote the test for which the maximum is achieved. As for the adaptive threshold design, the null distribution of L^* can be determined by repeating the analysis after permuting the treatment and control labels a thousand or more times. If the permutation statistical significance of L^* is $<0.05-\alpha_1$ (e.g., 0.02), then treatment T is considered superior to C for the subset of the patients positive for biomarker test k^*. The stability of the indicated set of patients who benefit from T (i.e., k^*) can be evaluated by repeating the computation of k^* for bootstrap samples of patients. This approach can be useful when the number of candidate biomarkers is small, as it should be by the time a phase III trial is initiated. Some of the adaptive enrichment designs (20) can also be employed in that setting with multiple biomarker candidates with or without known cut-points of positivity.

ADAPTIVE CLASSIFICATION BASED ON SCREENING CANDIDATE BIOMARKERS

Designs such as the "adaptive signature design" have been developed for adaptive multivariate classifier development and internal validation based on high dimensional genomic tumor characterization (24). This design employs a "learn and confirm" structure in which a portion of the patients are used to select the biomarker hypothesis, i.e., to develop an "indication classifier" which identifies the target population of patients in which the test treatment is most likely to be effective, and to use the remainder of the patients to test the treatment effect in that subset. The adaptive signature design does not modify eligibility criteria. It is adaptive in the sense that the treatment effect is tested in a single subset determined based on the clinical trial data but in a manner that separates classifier development from testing of treatment effect. This is dramatically different than the current practice of *ad hoc* analysis in multiple subsets with no control of type I error or in using the full dataset to both develop a classifier and to classify patients for purpose of hypothesis testing. Since the adaptive signature design does not use the patients on which the classifier was developed for the testing of the treatment effect, it thus avoids the inflation of type I error described by Wang et al. (25) for other approaches. Scher et al. described the use of the adaptive signature design for planning a pivotal trial in advanced prostate cancer (26). The key idea of the adaptive signature approach is to replace multiple significance testing based subset analysis with development and internal validation of a single "indication classifier" that informs treatment selection for individual patients based on their entire vector of covariate values.

The adaptive signature design approach is very general with regard to the methodology applied to the training set for identifying the single candidate subset in which treatment effect will be tested in the validation set. In many cases this can be accomplished by developing a model for predicting outcome as a function of treatment, selected biomarkers and treatment by biomarker interactions. In the original adaptive signature design paper this was accomplished by screening all the candidate biomarkers using predictive models that include the main effect of treatment, main effect of a single biomarker, and the corresponding interaction of that biomarker with treatment. Candidate markers which exhibited an interaction nominally significant at a pre-specified level were included in a final multivariate predictive model. A machine learning weighted voting model was used in the original paper to classify patients as either likely to benefit from the new treatment or not likely to benefit from the new treatment. The tuning parameters for this classifier were optimized by cross-validation in the training set. The multivariate model was then used to classify the patients in the validation set, and the treatment effect was evaluated in the subset of the patients in the validation set that were classified as likely to benefit from the new treatment based on the classifier developed in the training set.

Many other methods of classifier development can be employed using the training set. It is important to recognize, however, that one is not developing a prognostic classifier. The classifier is used to classify patients as likely to benefit from the new treatment. One could develop prognostic classifiers separately for the treatment and control groups using standard penalized regression methods and then classify patients based on which prognostic classifier predicts the better outcome. More commonly, however, single predictive models have been used based on screening candidate markers based on their univariate interaction with treatment. Matsui et al. (27) used their model to predict a continuous score reflecting the expected benefit for the new treatment relative to the control rather than just classifying patients into one of two subsets. Gu et al. (28) have developed a two-step strategy for developing a model for predicting outcome as a function of treatment and selected biomarkers. The biomarkers are selected using a group lasso approach in which the main effects of a biomarker are grouped with the interactions of that marker with treatments and can be used with two or more treatments.

Freidlin et al. (29) described further extensions of the adaptive signature approach. They use cross-validation to replace sample splitting of the trial into a training set and test set in order to increase the statistical power.

CONCLUSION

Recognition of the molecular heterogeneity of human diseases such as cancers of a primary site and the tools for characterizing

this heterogeneity presents new opportunities for the development of more effective treatments and challenges for the design and analysis of clinical trials. In oncology, treatment of broad populations with regimens that do not benefit most patients is less economically sustainable with expensive molecularly targeted therapeutics and less likely to be successful. The established molecular heterogeneity of human diseases requires the development of new approaches to use randomized clinical trials to provide a reliable basis predictive medicine (3, 4). This paper has attempted to review here some prospective phase III designs for the co-development of new therapeutics with companion diagnostics.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 September 2013; accepted: 11 December 2013; published online: 23 December 2013.

*Citation: Simon R (2013) Drug-diagnostics co-development in oncology. Front. Oncol. **3**:315. doi: 10.3389/fonc.2013.00315*

This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology.

frontiers in ONCOLOGY

REVIEW ARTICLE
published: 01 April 2014
doi: 10.3389/fonc.2014.00058

Will the requirement by the US FDA to simultaneously co-develop companion diagnostics (CDx) delay the approval of receptor tyrosine kinase inhibitors for RTK-rearranged (*ROS1*-, *RET*-, *AXL*-, *PDGFR*-α-, *NTRK1*-) non-small cell lung cancer globally?

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The discovery of anaplastic lymphoma kinase (*ALK*) rearrangement in non-small cell lung cancer (NSCLC) in 2007 and the approval of crizotinib for the treatment of advanced *ALK*-rearranged NSCLC in 2011 represents a landmark in the development of targeted oncology therapy. The approval of crizotinib was accompanied simultaneously by the approval of the Vysis (Abbott Molecular) break-apart fluorescence *in situ* hybridization (FISH) test as the companion diagnostic (CDx) test to detect *ALK* rearrangement. Pfizer, the manufacturer of crizotinib, sponsored the screening of thousands of patients and the standardization of the *ALK* FISH test as part of the approval process for crizotinib, a first in class ALK inhibitor. Many pharmaceutical companies are now using the Food and Drug Administration (FDA)-approved *ALK* FISH assay to enroll patients onto trials for their own respective ALK inhibitors. In essence they are "piggy-backing" on the FDA-approved *ALK* FISH assay without having to pay for the development of a CDx, nor screening for *ALK*-rearranged NSCLC patients in the protocols because screening for *ALK* rearrangement is now the standard of care in NSCLC after the approval of crizotinib. Since 2007, rearrangement in more receptor tyrosine kinases (RTKs) such as *ROS1*, *RET, AXL*, *PDGFR*-α, *and NTRK1* have been discovered in NSCLC but the incidence of each subtype of RTK-rearranged NSCLC is quite rare. Crizotinib has now demonstrated significant clinical activity in *ROS1*-rearranged NSCLC patients. Whether crizotinib will gain official FDA approval for use in *ROS1*-rearranged NSCLC, on the other hand, remains unclear as there is no test for *ROS1*-rearrangement currently being developed to support US FDA approval as a CDx. This may be due in part to the fact that the full cost associated with the development of a pre-market approved-approved CDx must be borne by the company seeking the first drug approval in a new indication. Given the low incidence of *ROS1*-rearrangement in NSCLC, and the availability of crizotinib in most countries, a more cost-effective way is for crizotinib to gain compendium listing for *ROS1*-rearranged NSCLC in treatment guidelines. However, without a formal indication from the FDA, a drug cannot be marketed for off label use, it is unlikely that payers public or private will routinely pay for molecular testing for *ROS1*-rearrangement in NSCLC let alone reimburse off label use of crizotinib. Similarly, several marketed tyrosine kinase inhibitors (TKIs) in the US (sorafenib, sunitinib, vandetanib, cabozantinib, regorafenib) are potent RET inhibitors *in vitro*. It does not make sense for any one pharmaceutical company to shoulder the full cost of developing a particular CDx for *RET*-rearranged NSCLC where, once approved, it may be used by other pharmaceutical companies to gain addition labeling approval for their own RET inhibitors. Thus, the requirement by the US FDA that a specific CDx have to be co-developed and standardized for each of the molecular subtype of NSCLC as part of the drug approval process, while prudent, may have the un-intended consequence of deterring clinical development of these TKIs in these very rare molecular subsets of NSCLC. While we all march to the drumbeat of precision cancer medicine, the stringent requirement of co-development CDx for each molecular subtype of solid tumor may inadvertently make this goal substantially more difficult to achieve.

Keywords: companion diagnostics, *ALK*-rearranged NSCLC, *ROS1*-rearranged NSCLC, *RET*-rearranged NSCLC, fluorescence *in situ* hybridization, immunohistochemistry, next generation sequencing, reverse transcription-polymerase chain reaction

INTRODUCTION

Achieving personalized medicine is the "holy grail" in oncology. The approval of crizotinib in the US, an anaplastic lymphoma kinase (ALK)/ROS1/MET multi-targeted tyrosine kinase inhibitor (TKI), merely 4 years after the discovery of rearrangement in ALK in non-small cell lung cancer (NSCLC) represented a landmark in oncology drug development and a significant step toward the goal of personalized medicine in oncology (1). The approval of crizotinib was accompanied the simultaneous approval of the Vysis (Abbott Molecular) break-apart fluorescence *in situ* hybridization (FISH) companion diagnostics (CDx) assay by the US Food and Drug Administration (FDA) for the detection of *ALK* rearrangement in NSCLC. The success of crizotinib has shone a bright spotlight on the existence of molecular subsets of NSCLC and other epithelial malignancies that are driven by rearrangement in receptor tyrosine kinases (RTKs) and heralded the era of RTK rearrangement in solid tumor oncology. Since 2007 other RTK-rearrangements in NSCLC have been discovered (**Table 1**). Concurrently, various diagnostic tests besides FISH have been offered by major commercial diagnostic companies in the US to detect the different RTK-rearrangements. Given the rarity of RTK rearrangement in NSCLC and the requirement by US FDA to develop an analytically and clinically validated CDx for approval of TKIs against each RTK-rearranged molecular cohort, challenges abound in persuading many pharmaceutical companies to pursue a simultaneous registration strategy. We will review the lessons learned from the development of crizotinib for *ALK*-rearranged NSCLC where several second generation ALK inhibitors are in now development due to the existence of an FDA-approved CDx, the ongoing challenges in gaining additional FDA approval for crizotinib in the treatment of *ROS1*-rearranged NSCLC due to a lack of an approved CDx for *ROS1*-rearranged NSCLC, the immense challenges in gaining approval for any currently marketed TKI that are also potential RET TKI for the treatment of *RET*-rearranged NSCLC due to again the lack of an FDA-approved CDx for *RET* rearrangement (**Table 2**). Additionally, we will discuss whether the first FDA-approved CDx is the optimal CDx going forward given the inevitability of technology obsolescence coupled with the exponential gain in knowledge in the understanding of these subsets of molecularly defined NSCLC. Finally, we speculate that if the current challenges of co-CDx approval are not overcame how the development of precision cancer medicine may be impeded.

THE DISCOVERY OF RECEPTOR TYROSINE KINASE-REARRANGED (*ALK-*, *ROS1-*, *RET-*, *AXL-*, *PDGFR-α-*, *NTRK1-*) NSCLC

All the RTK-rearrangements identified in NSCLC occur in genes of the human RTK family, which consists of 58 members (11). The discovery of *ALK* rearrangement in NSCLC in 2007 was significant because prior to the discovery it was believed that gene fusions especially involving RTK rearrangement were believed to be rare in epithelial tumors (12). It is abundantly clear that each subtype of RTK-rearranged NSCLC is itself a heterogeneous disease made up many different (and yet to be discovered) fusion partners translocated to the same RTK (**Table 1**). The complexity within each molecular subtype of RTK-rearranged NSCLC have implications on the CDx. Ideally a CDx should be technically simple and/or be easily standardized, cost-effective, but also provide "forward-looking" information such as the exact fusion variant with at the exact breakpoint so that subtle differences among the various fusion variants within each molecular subtype of RTK-rearranged NSCLC can be elucidated.

Rearrangement of *ROS1* in NSCLC was discovered contemporaneously in 2007 by one of the two groups that discovered *ALK* rearrangement (13). *ROS1* shares extensive amino acid sequence homology with ALK in particular within the kinase domain making ROS1 a potential target for ALK inhibitors (14). Prior to 2007, *ROS1*-rearrangement was discovered in glioblastoma multiforme (15) and subsequently has been discovered in other major epithelial tumor types including gastric (16) and colorectal adenocarcinoma (17).

The *RET* (rearranged during transfection) proto-oncogene was first identified in 1985 through transfection of NIH3T3 cells with human lymphoma DNA (18). RET rearrangement has also been well characterized in thyroid cancer (19). Since 2012, multiple groups using various techniques published the rearrangement of RET in NSCLC with four identified fusion partners so far (*KIF5B-CCDC6-*, *NOCA4-*, *TRIM33-*) (2) (**Table 1**).

Rearrangement of the tropomyosin-related kinase gene (TRKA) was first biologically characterized in 1986 in a colorectal carcinoma patient (20), when tropomyosin was found to be fused to an unknown DNA sequence that likely codes for a transmembrane RTK (*TPM3-TRKA*) (20). The normal function of TRKA is the receptor for neurotrophins and is responsible for differentiation into subtypes of sensory neurons. TRKA has been renamed as neurotrophic tyrosine receptor kinase 1 (NTRK1) as it is one of three members of NTRK family (21). In 2013, rearrangement in NTRK1 was reported in NSCLC involving fusion partners with CD74 and MPRIP as fusion partners (*CD74-NTRK1*, *MPRIP-NTRK1*) (4). Screening a panel of NSCLC that are pan-negative for oncogenic driver mutations, they found 3 out of 91 (3.3%) were positive for *NTRK1* rearrangement. Cell-based and xenograft assays using NTRK1 inhibitors in NTRK1 transformed cells led to inhibition of cellular proliferation and tumor shrinkage, respectively, indicated *NTRK1* rearrangement are indeed a driver mutation in NSCLC (4). Of note similar to RET, rearrangement of *NTRK1* has been described in thyroid cancer (*TPM3-NTRK1*, *TPR-NTRK1*, *TFG-NTRK1*) (22).

AXL, termed from the Greek word anexelekto, or uncontrolled, was identified initially as a transforming oncogene in two chronic myelogeneous leukemia (CML) patients in 1991 (23). In 2012, *AXL* was found to be fused to MAP3K12 binding inhibitory protein 1 (MBIP) resulting in *AXL-MBIP* fusion variant by whole genome sequencing (WGS) (3). In the same study, Seo et al. also discovered the platelet derived growth factor receptor-alpha (*PDGFR-α*) was fused to SR-related CTD-associated factor 11 (*SCAF11-PDGFR-α*) in NSCLC (3). Prior to that, rearrangement in *PGDFR-α* was found in myeloid and lymphoid neoplasms with esinophilia where *PDGFR-α* is fused to Flip1-like 1 gene (FIP1L1) (*FIP1L1-PDGFR-α*) (24). Interesting aberrantly activation by phosphorylation of *PDGFR-α* was demonstrated in one cell line (H1703) and several patient samples in 2007 but no rearrangement was discovered (13). In summary, many of the RTK-rearrangements in NSCLC were discovered in other tumors but because of the success of crizotinib

Table 1 | Characteristics of RTK rearrangement in NSCLC.

RTK rearrangement	Year identified	Fusion partners	Estimate prevalence (%)	Methods of initial identification	Select reference
ALK	2007	EML4-, KIF5B-, KCL-, TFG-	~5–8	Tumor DNA transfection, Phospho-kinase activation	Ou et al. (1)
ROS1	2007	CD74-, SDC4-, SLC34A2-, TPM3-, FIG-, KDEL2-, CCDC6-, LRIG3-, ERZ-	~2	Phospho-kinase activation	Gainor and Shaw (2)
RET	2012	KIF5B-, CCDC6-, NOCA4-, TRIM33-	~2	FISH, NGS, WGS	Gainor and Shaw (2)
AXL	2012	MBIP-	NA	WGS	Seo et al. (3)
PDGFR-α	2012	SCAF11-	NA	WGS	Seo et al. (3)
NTRK1	2013	CD74-, MPRIP-	~3[a]	FISH, NGS	Vaishnavi et al. (4)

[a] *3.3% in ALK, ROS1, RET negative NSCLC.*

the discovery of these RTK-rearrangements in NSCLC has drawn increased attention to these RTKs in all tumor types (25).

ALK INHIBITORS FOR THE TREATMENT OF *ALK*- AND *ROS1*-REARRANGED NSCLC

While crizotinib is the first and only ALK inhibitor approved for the treatment of advanced *ALK*-rearranged NSCLC since August 2011, the majority of patients invariably progress on crizotinib with a median progression-free survival of about 8 months (26). The incorporation of break-apart ALK FISH as the FDA-approved CDx for detection of *ALK* rearrangement through the approval of crizotinib has provided a new standard of care with an established assay to screen for and enroll these *ALK*-rearranged NSCLC patients onto clinical trials of these ALK inhibitors. Pfizer, the manufacturer of crizotinib, engaged a diagnostic company to support both the development and technical validation of the *ALK* FISH CDx. In this case, Abbott Molecular sponsored the *ALK* FISH screening test and the validity of the CDx and the regulatory approval of the CDx as well as all screening of patients, to support the drug approval but Pfizer paid for everything Abbott Molecular. In retrospect, Pfizer essentially paved the way for competitors to more easily develop follow-on ALK inhibitors by establishing the clinical validity of a CDx test and screening for *ALK*-rearranged NSCLC patients. This realization, we believe has important implications on how the CDx for the other unique RTK-rearranged NSCLC may be developed by pharmaceutical companies.

Crizotinib has also shown significant clinical activity in *ROS1*-rearranged NSCLC due to the homology between the kinase domain (27). As part of the original phase I crizotinib trial (PROFILE1001, NCT00585195), the assay for the trial to detect *ROS1*-rearrangement is a locally developed laboratory-based test and no formal CDx is being developed for FDA approval in conjunction with the trial. In order for Pfizer to gain formal FDA approval for crizotinib in *ROS1*-rearranged NSCLC, Pfizer may have to sponsor another large scale trial and more importantly pay for the screening and analytical and clinical validation of a *ROS1* CDx (likely be FISH again) so that a CDx can be submitted simultaneously for FDA approval in support for the clinical activity of crizotinib in *ROS1*-rearranged NSCLC. However, once a CDx for *ROS1*-rearrangement is approved by the US FDA, other pharmaceutical companies can take advantage of the existence of an FDA-approved ROS1 CDx to develop their own ROS1 inhibitors similarly to the situations for current ALK inhibitors in clinical development. Given the low incidence of *ROS1*-rearranged NSCLC (~2%), Pfizer or other pharmaceutical companies is unlikely to make this investment given crizotinib is already available in many countries. Furthermore, although many Clinical Laboratory Improvement Amendments (CLIA)-certified commercial diagnostic companies in the US are offering *ROS1*-rearrangement testing [either by break-apart FISH, reverse transcription-polymerase chain reaction (RT-PCR), or even next generation sequencing (NGS)], without an official indication from the US FDA, screening for *ROS1*-rearrangement among community oncologists in the US will not be a common practice. Without an official FDA indication of crizotinib for *ROS1*-rearranged NSCLC, even with the endorsement of the National Comprehensive Cancer Centers Network (NCCN) guidelines, insurance companies may not pay for crizotinib for the few *ROS1*-positive NSCLC patients, even if their oncologists prescribe it. Furthermore, without an FDA indication for *ROS1*-rearranged NSCLC, the research of *ROS1*-rearrangement in other major epithelial tumor types such as colon (17) and gastric cancer (16), the cost of co-developing a companion diagnostics for *ROS1*-rearrangement will dissuade a lot of pharmaceutical companies to pursue a registration strategy in any *ROS1*-rearranged tumors even if they have potent ROS1 inhibitors in the pipeline.

WILL A RET INHIBITOR EVER BE FORMALLY APPROVED BY THE US FDA FOR *RET*-REARRANGED NSCLC AND WHAT IS THE IMPLICATION IF THE ANSWER IS NO?

We ask this question because the clinical reality of *RET*-rearranged NSCLC is even more relevant in illustrating the central theme of this perspective. There are currently at least six marketed TKIs (regorafenib, cabozantinib, ponatinib, sunitinib, sorafenib, vandetanib) in the US that are also potent *in vitro* RET inhibitors (**Table 2**). Under the current US FDA regulations, manufacturers of any one of the above marketed TKIs who wants to gain an additional approval for treatment of *RET*-rearranged NSCLC will have

Table 2 | List of potential RET inhibitors potentially for the treatment of RET-rearranged NSCLC.

Compound	Trade name	Manufacturer	*In vitro* kinase IC_{50} (nM) against RET	*In vitro* cellular kinase IC_{50} (nM) against RET	*In vitro* IC50 (nm) against RET V804 mutant	Other targets	Approved indications in the US	In clinical trial for RET-rearranged NSCLC	CDx used to detect *RET* rearrangement in NSCLC trials
Regorafenib (5)	Stivarga	Bayer	1.5	~10	NR	VEGFR1-3, KIT, RAF-1, BRAF, BRAFV600E, PDGFR-β	Treatment refractory colorectal adenocarcinoma	No	N/A
Ponatinib (6)	Iclusig	ARIAD	7	0.7–11	12	Bcr-abl, FGFR1-4,	TKI resistance CML or Ph + ALL	Yes[a] NCT01813734	FISH, NGS
Cabozantinib (7)	Cometriq	Exelixis	5.2	27–85	>5000	VEGFR2, c-MET	Medullary thyroid cancer	Yes NCT01639508	FISH, NGS
Lenvatinib (E7080) (8)	N/A	Eisai	1.5	48 (CCDC6-RET)	NR	VEGFR1-3, FGFR1-3, PDGFR, c-kit	N/A	Yes NCT01877083	NGS
Sunitinib (6)	Sutent	Pfizer	30	40–164	55	PDGFR, VEGFRs, c-kit, FLT-3	RCC, GIST, unresectable/ metastatic PNET	Yes NCT01829217	FISH, NGS
Sorefenib (9)	Nexaavar	Bayer	47	~20–50	55	Raf, PDGFR, VEGFR2, VEGFR3, c-kit,	HCC, RCC,	No	N/A
Vandetanib (10)	Caprelsa	AstraZeneca	100	NR	NR	VEGFR, EGFR	Medullary thyroid cancer	Yes NCT01823068	FISH

[a] *Currently on hold.*

N/A, not applicable; NR, not reported.

PDGFR, platelet derived growth factor receptor; NGS, next generation sequencing; PNET, pancreatic neuroendocrine tumor; VEGFR, vascular endothelial growth factor receptor.

to pay for the screening for thousands of NSCLC patients and the development of a *RET*-rearrangement CDx. Again given the low incidence of *RET*-rearranged of NSCLC (~2%) and the potential crowded market for RET inhibitors, it is unlikely manufacturer of any one of the six potential marketed RET inhibitors will sponsor such as a trial, lest it will allow competitors to piggyback on the CDx to gain approval of their TKIs without shouldering the cost for patient screening and developing an approvable CDx. This is currently, the case as all the clinical trials in these marketed TKIs are investigator-initiated trials with a diverse platforms to screen for *RET* rearrangement (**Table 2**). Indeed, preliminary clinical activity of cabozantinib in three *RET*-rearranged NSCLC patients has been recently published (28). The exception is the manufacturer of lenvatinib (E7080) (Eisai Company, Ltd.) who is sponsoring a trial of lenvatinib in *RET*-rearranged NSLCL primarily in Asia using NGS as the primary CDx (NCT01829217) (**Table 2**). Without a US FDA-approved RET CDx, not only will potential RET inhibitors not gain US FDA approval to treat *RET*-rearranged NSCLC but other *RET*-rearranged malignancies such as thyroid cancer (19) or chronic myelomonocytic leukemia (CMML) (29).

Going forward, many small molecular inhibitors are being developed against *AXL* (30) and *NTRK1* (31, 32). Additionally, imatinib has shown excellent clinical activity against myeloid and lymphoid malignancies harboring *FIP1L1-PDGFR-α* rearrangement (33). Thus, achieving the goal of precision cancer medicine hinges on formal approval of these inhibitors to treat these various rare but diverse molecularly defined and driven malignancies and the requirement to co-develop a CDx may be a huge impediment to achieving this goal.

IS THE FIRST APPROVED CDx THE BEST CDx CONSIDERING THE ISSUES OF COST-EFFECTIVENESS, KNOWLEDGE ADVANCEMENT, AND TECHNOLOGY OBSOLESCENCE?

The approval of the Abbott Vysis break-apart FISH assay by the FDA as the CDx for the diagnosis of *ALK*-rearranged NSCLC seemed to have established break-apart FISH as the lead method platform to diagnose RTK rearrangement in NSCLC. However, break-apart FISH is probably "the worst of both worlds" as a CDx platform. There are three major criteria that have to be satisfied for a break-apart FISH to be considered positive: (1) a minimum of 50 cells have to be counted; (2) signals are considered "break-apart" when they are separated by at least two diameter in length OR only the 3′ signal is present; (3) at least 15% of the cells have to contain the break-apart signals. Polysomy is common in *ALK*-rearranged lung cancer tumor (34) thus, identifying all these criteria requires technical expertise and expert interpretation and is labor-intensive and time consuming. Additionally, FISH is prohibitively expensive as a mass screening method for many countries. Finally, FISH will not identify the specific fusion partner to the rearranged RTK gene. As our knowledge about RTK-rearranged NSCLC grows, it is highly likely that different RTK fusion variant will have different clinicopathologic characteristics such as extent of disease, site of metastasis, and differential response to TKIs (35), which required even more tailored treatment in the future. In summary, FISH is neither an inexpensive mass screening CDx nor does it lead to further understanding of the pathogenesis of RTK-rearranged NSCLC.

In contrast, ALK protein is only expressed in tumor tissue due to transcriptional activation from the promoter of the 5′-fusion partner to ALK but not in normal tissue and can be easily detected by immunohistochemistry (IHC). IHC is inexpensive and easily performed by all pathologists. Furthermore, ALK IHC has been demonstrated to show high concordance to ALK FISH (36). Since October 2012, IHC (Ventana automated staining system using D5F3 antibody from Cell Signaling Inc.) has been approved in the European Union (EU) as a CDx to detect *ALK* rearrangement along with break-apart FISH. This automated ALK IHC staining platform has shown extremely high sensitivity and specificity to ALK FISH (37). In September 2013 China approved the same method approved in EU to detect *ALK* rearrangement.

Immunohistochemistry has been used to detect *ROS1*-rearrangement in NSCLC and the sensitivity and specificity of ROS1 IHC is found to be 100 and 92%, respectively (38). Thus, it is likely with further refinement, IHC will likely be widely used to detect *ROS1*-rearrangement. On the other hand, RET is highly expressed in normal tissue and the sensitivity of RET IHC is low and thus, IHC may not be an ideal CDx to diagnose *RET* rearrangement (39). Thus, while IHC is a standard pathology procedure and cheaper than FISH, it is not applicable to all the different RTK-rearrangements depending on the normal expression pattern of the RTK in that particular tumor type. Much remain to be discovered on the expression level of *TRK1*-, *AXL*-, and *PDGFRα*- fusion proteins in NSCLC before we can really assess the utility of IHC in the detecting of these newly discovered molecular subtypes of RTK-rearranged NSCLC.

Reverse transcription-polymerase chain reaction is another commonly utilized research technique to detect RTK rearrangement. RT-PCR is highly specific and can be easily performed in standard diagnostic laboratories. However, most of the RT-PCR studies require large volume of tumor tissue snapped frozen from surgical resection. In daily oncology practice, the vast majority of the NSCLC are diagnosed from fine or core needle biopsy from which the tissue is placed in formalin instead of snap frozen at −80°C. RNA is not easily preserved in formalin-fixed tissues and thus RT-PCR may not be technically feasible in many of the samples. Also given that each unique molecular subtype of RTK-rearranged NSCLC has many different fusion variants; in order to identify all the known fusion variants the PCR has to contain primers to all the fusion partners. Any un-reported/un-discovered fusion partner will be missed by RT-PCR. In the case of *ROS1*-rearrangement, at least nine sets of primers for the nine reported fusion partners have to be present in the RT-PCR. Therefore, although RT-PCR has been commercialized in the US to detect RTK-rearranged NSCLC (40), it is not a widely adopted CDx and unlikely to gain global acceptance.

Next generation sequencing is a broad term that generally describes the massively parallel sequencing approach and employing various detection methods on a panel of genes that are altered in cancer. Many NGS panels of varying number of gene are now being offered commercially. For example, Foundation Medicine Inc., is offering a 236 gene test that can detect mutations, copy number alterations, and 19 gene rearrangements that has been used commercially used to detect new *RET* rearrangement in

an investigator-initiated trial (28) or previously undetected *ALK* rearrangement (41).

Advances in the understanding of neoplastic diseases couple with technical advancement in the field of diagnostic tests raise the ongoing issue of technology obsolescence supporting the original FDA-approved test. Technology obsolescence will invariably poses a significant problem with time because one particular technology/diagnostic platform (i.e., FISH) is essentially linked to drug labeling by the FDA. With time that one specific diagnostic platform may turn out to be expensive, highly operator dependent with a steep learning curve, not easily automatable, and provide scant clinical information (e.g., FISH does not provide the fusion partner nor the break-point, which may be important in underlying the clinicopathologic and natural history of that particular RTK rearrangement). The ideal future CDx should be able to pinpoint chromosomal breakpoint and to identify the various fusion partners to a particular RTK rearrangement so that, we can continue to advance our molecular understanding of oncology in order to refine our approach to personalized medicine.

However, to get a different CDx platform approved by the FDA will again incur significant expense not only in standardization and validation of the new CDx but the cost of conducting a clinical trial "reinventing" the original approval process.

SAMPLE SURVEY OF THE APPROVED INDICATIONS FOR CRIZOTINIB OUTSIDE THE US

Crizotinib received conditional approval in the EU in July 2012 for previously treated ALK-positive NSCLC with the recommendation that a validated test for *ALK* rearrangement be used. Similarly crizotinib was approved in Singapore in 2013 for the treatment of locally advanced or metastatic *ALK*-rearranged NSCLC detected by an accurate and validated test. However, no one particular CDx (such as FISH) was specified by the approval in both EU and Singapore. Granted that in EU the approval of medicines and CDx are coordinated by two different agencies (42). Indeed, since October 2012, Vetana ALK IHC has been approved as a CDx for *ALK* rearrangement also. In Korea (2012), Japan (2012), and Australia (2013), crizotinib was approved for treatment of *ALK*-rearranged NSCLC without mention of the detection method. Granted by 2012, there is plentiful data supporting high concordance FISH and IHC (36) or even NGS (41) thus it is not necessary to pigeonhole a drug approval to one particular CDx. However, without the initial US FDA approval of crizotinib and the advance in knowledge over the intervening years it is likely that "relaxed" CDx requirement will not be possible in many countries. Thus, approval of the US FDA remains the gold standard for the drug regulatory agencies and authorities in many countries.

CONCLUDING PERSPECTIVES

Many of the RTKs discussed in this perspective were discovered in 1980s as transformed oncogenes due to elegant basic science research. It has been more than 30 years since then to now where we are at the cusp of realizing precision cancer medicine by successfully translating these discoveries to therapeutic approvals and finally bearing fruit of all the research funding for the benefit of patients. The successful launch of crizotinib has been an inspiring example of this development.

The technologies to screen for these RTKs in all tumors are commercially available; inhibitors to these RTKs are either approved for other indication or in early clinical development. Because of the rarity of these RTK-rearrangements, the cost of sponsoring a registration trial for a particular TKI and simultaneous development of a CDx is prohibitively expensive and clinical progress is being delayed due to reluctance of pharmaceutical companies to pursue such narrow indications in rare disease populations.

One attractive though organizationally challenging solution may be to foster a collaboration of government, pharmaceutical companies, and diagnostic companies pooling resources together to an independent consortium to establish analytical and clinical validity of CDx platforms for detection of RTK-rearrangements and potentially other cancer genes. The US FDA may then approve these CDx platforms such as FISH, IHC, or NGS for each or several RTK-rearrangements and then allowing pharmaceutical companies to sponsor the trials and select any of the CDx platforms to demonstrate clinical benefit. This will alleviate the burden of simultaneously developing a CDx that can then be "piggybacked" by other pharmaceutical companies developing their own inhibitors. Additionally, this will eliminate potential conflict of interest as some global pharmaceutical companies also own major diagnostic companies (i.e., Ventana Medical Systems by F. Hoffmann-La Roche, Genoptix by Novartis) where one particular diagnostic platform may be favored by one pharmaceutical company due to technological knowhow and/or existing patents.

Short of industry-wide cooperation, regulatory policy may be used to lower regulatory burdens and create a more favorable incentive structure for therapeutic and diagnostics companies pursuing targeted therapy and CDx development. For instance, to reduce CDx costs, certain CDx quality systems and validation requirements may be simplified or deferred to the post-approval period, given appropriate risk determination. And as above, some assays may be approvable based on analytical validation data alone, decoupling diagnostic from therapeutic development decisions and thus streamlining coordination.

The requirement for co-development and co-approval of CDx in order to get TKIs approved against these RTK (*ROS1*, *RET*, *NTRK1*, *AXL*, *PDGFR*-α) rearrangement lung cancer represents the daunting challenge to successfully translate decades of basic science research into benefit of cancer patients. However, the successful approval of TKIs to treat *ROS1*-, *RET*-, *NTRK1*-, *PDGFR*-α, and *AXL*-rearranged NSCLC is vitally important as it sets the example for approval of TKIs to treat the same RTK-rearranged common epithelial tumors such as colon, gastric, and breast cancers (25). Using NSCLC as a tumor example, we wish this perspective contributed to the ongoing in-depth discussions about how to optimally and expeditiously develop TKIs to receive US FDA approval in the current regulatory environment where co-development and co-approval of a CDx is required for a drug in other TK-driven cancers.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

*Received: 04 January 2014; accepted: 11 March 2014; published online: 01 April 2014. Citation: Ou S-HI, Soo RA, Kubo A, Kawaguchi T and Ahn M-J (2014) Will the requirement by the US FDA to simultaneously co-develop companion diagnostics (CDx) delay the approval of receptor tyrosine kinase inhibitors for RTK-rearranged (ROS1-, RET-, AXL-, PDGFR-α-, NTRK1-) non-small cell lung cancer globally? Front. Oncol. **4**:58. doi: 10.3389/fonc.2014.00058*

This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology.

frontiers in ONCOLOGY

ORIGINAL RESEARCH ARTICLE
published: 05 February 2014
doi: 10.3389/fonc.2014.00013

Customizing the therapeutic response of signaling networks to promote antitumor responses by drug combinations

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Drug resistance, *de novo* and acquired, pervades cellular signaling networks (SNs) from one signaling motif to another as a result of cancer progression and/or drug intervention. This resistance is one of the key determinants of efficacy in targeted anti-cancer drug therapy. Although poorly understood, drug resistance is already being addressed in combination therapy by selecting drug targets where SN sensitivity increases due to combination components or as a result of *de novo* or acquired mutations. Additionally, successive drug combinations have shown low resistance potential. To promote a rational, systematic development of combination therapies, it is necessary to establish the underlying mechanisms that drive the advantages of combination therapies, and design methods to determine drug targets for combination regimens. Based on a joint systems analysis of cellular SN response and its sensitivity to drug action and oncogenic mutations, we describe an *in silico* method to analyze the targets of drug combinations. Our method explores mechanisms of sensitizing the SN through a combination of two drugs targeting vertical signaling pathways. We propose a paradigm of SN response customization by one drug to both maximize the effect of another drug in combination and promote a robust therapeutic response against oncogenic mutations. The method was applied to customize the response of the ErbB/PI3K/PTEN/AKT pathway by combination of drugs targeting HER2 receptors and proteins in the down-stream pathway. The results of a computational experiment showed that the modification of the SN response from hyperbolic to smooth sigmoid response by manipulation of two drugs in combination leads to greater robustness in therapeutic response against oncogenic mutations determining cancer heterogeneity. The application of this method in drug combination co-development suggests a combined evaluation of inhibition effects together with the capability of drug combinations to suppress resistance mechanisms before they become clinically manifest.

Keywords: anti-cancer combination therapy, cancer drug resistance, pathway engineering, signaling networks, pertuzumab, PI3K/PTEN/AKT

INTRODUCTION

Anti-cancer combination therapies are an increasingly promising way to better treat patients, offering an increase in efficacy of drugs, and a means to overcome/avoid resistance to targeted drug therapy. An evidence base of successes of multidrug strategies in anti-cancer therapy is growing yearly, but typically the mechanism of synergy is not entirely understood. To promote a more directed and systematic development of combination therapies, it is necessary to clearly elucidate the underlying mechanisms that drive the advantages of drug combination effect. Exploration of these mechanisms can impact significantly on the determination and validation of advanced combination of targets in novel drug development strategies such as drug combination co-development (1) and drug-diagnostics co-development (2). With respect to a drug combination co-development strategy, novel compounds should be developed in a combinatorial context and not independently in order to gain those proven integrative benefits with respect to tumor growth inhibition, drug resistance, and toxicity. In the drug–diagnostic co-development model, the drug and the diagnostic assay are developed in conjunction. Companion diagnostic (CDx) assays as well as clinical trial strategies in the case of drug combination therapy should be adapted to multiple biomarkers and different drug combinations (2). Considering the risk of increasing toxicity by the use of drug combination therapy, the US food and drug administration (FDA) released drug co-development guidance, which proposed stringent regulatory recommendations for the use of combination strategies (1, 3). According to these recommendations, there should exist a strong biological rationale for the use of the combinations and proven significant advantages over the use of the drugs as individual agents.

Rational determination and validation of advanced targets for drug combination therapy are the main challenges for these

strategies. The inherent complexities of signaling networks (SNs), including crosstalk logic and redundancy in their topology (4, 5) limit the relevance of genotype-based screening to identify and validate optimal drug combination targets among several hundred possible targets. In the face of these challenges, we need more sophisticated methods to design effective combination therapies (1).

Systems biology and computational bioinformatics may offer a key role in advancing drug combination co-development. These technologies are being developed to support rationalized screening of effective drug combinations, and to formulate quantitative criteria for drug combination target validation and specific properties of each drug in a combination context and in the face of different mutational statuses of different cancers (6–9). Such developments are motivated by the potential to reduce the size, duration, and cost of clinical trials as well as to accelerate regulatory approval. Our own studies to support drug combination co-development are described here. Our method is based on analysis of the response of a cellular SN to drug intervention, and its modification by drug combination to achieve high efficacy and reduce drug resistance potential. The approach developed here is founded on the two most attractive advantages of drug combination well established recently: (i) a drug combination suppresses *de novo* or acquired resistance to one of the drugs in the combination – a resistance suppression effect; and (ii) one drug in a combination sensitizes therapeutic response to a second drug – a sensitizing effect.

Resistance suppression effects arising from drug combination are used widely in clinics now, by manipulating pathway dynamics or by slowing down the cancer evolution. Some examples of pathway manipulations are summarized in Ref. (1) targeting different domains of the same protein with two drugs [for example, trastuzumab combined with pertuzumab for human epidermal growth factor receptor, HER2, in breast (10) and ovarian (11, 12) cancers]; horizontally targeting parallel signaling pathways [for example, combined MEK and PI3K/AKT inhibition (13, 14)], and vertically targeting up- and down-stream pathways in one SN [for example, targeting BRAF and MEK, HER2, and PI3K, PI3K/AKT and mTOR (15–17)]. An evolutionary approach (adaptive therapy) to treat drug resistance has been suggested, with the aim of lowering the cancer cell's capacity to evolve resistance mechanisms through adaptive combination therapy in order to hinder the emergence of resistance types (18). Beyond these approaches, the combination of chemotherapy and molecular targeted therapy demonstrates a complementary mechanism of efficacy and may suggest a route for next-generation cancer therapy (10, 19, 20). For example, a clinical trial of HER2-positive breast cancer progression following HER2 inhibitor trastuzumab therapy showed that trastuzumab in combination with chemotherapeutic agent (capecitabine) was more effective than capecitabine alone. It would seem that trastuzumab sensitizes cell response to other drugs despite the fact that cells are refractory to trastuzumab and that there is therapeutic benefit to continue trastuzumab therapy in combination with other drugs beyond progression (21).

The sensitizing effects of drug combinations are well established in combined therapies targeting different pathways, such as the PI3K/PTEN/AKT and MAPK networks (13, 14); EGFR, DNA-damaging apoptotic signaling pathways (22) as well as in drug combinations inhibiting different nodes in the same pathway: PI3K/PTEN/AKT/mTOR (23–25), Ras/RAF/MEK/ERK (26, 27). However, there are longer-term implications arising from drug combinations since they may lead to different mutations and negatively impact resistance potential. For example, comparison of pre- and post-trastuzumab treatment of tissue samples in metastatic HER2-amplified breast cancer from patients progressing on trastuzumab allowed determination of mutations in *PIK3CA* and *PTEN* arising during treatment (4). A significant reduction, or full loss, of PTEN expression was observed in trastuzumab refractory metastatic tumors compared to the cohort untreated by trastuzumab. It was suggested that PTEN expression reduction was acquired as a result of trastuzumab therapy, leading itself to trastuzumab resistance (4). In contrast, treatment by everolimus, an mTOR inhibitor, did not increase mutational load in pre/post treatment samples of renal carcinomas (28). Moreover, this phenomenon is context specific: for example, abiraterone alone and given in combination with other drugs to control side effects contributes to resistance by activating mutations in the hormone receptor genes; however, a different combination including abiraterone was reported to delay drug resistance (29).

These examples highlight the complexities of combination drug therapy design, including the change in sensitivity to mutation and resistance potential through combination action, and the attending challenges associated with validation. To help overcome these complexities and challenges, we propose a method for the *in vitro*/*in vivo*/*in silico* validation of the targets of drug combinations and quantitative estimation of the perturbations induced by drugs in SNs. Our method is based on the study of drug response characteristics of (i) a SN and its modification by different oncogenic mutations and drug action; and (ii) perturbation of SN sensitivity to drug action induced by mutations. Using sensitivity analysis (SA) of this signaling response, we show that inhibition of various targets by the first drug significantly sensitizes SN response to both compensating mutations leading to resistance and the second drug in combination. We present a scheme for customizing the SN response through drug combination to enhance the robustness of the therapeutic response due to weakening of the drug-sensitizing effect to compensation mutations. To demonstrate the method and to study in detail the drug/mutation modification of SN response, we used the kinetic model of drug combination targeting HER2 receptors and vertical PI3K/PTEN/AKT signaling pathway (30–32).

MATERIALS AND METHODS

The method is based on the analysis of input/output (I/O) responses of a cellular SN, $R_{SN} = C_{out}$ (C_{in}, P_{SN}, D, t), which describes the relation between output signal C_{out} and input signal C_{in} (see scheme in **Figure 1**). R_{SN} depends on molecular parameters, P_{SN}, of the SN components: specifically, kinetic parameters of the proteins/receptors, $K = k_1, \ldots, k_n$ (dissociation constants, catalytic rates), and their expression levels, $E = e_1, \ldots, e_m$. R_{SN} also depends on the concentrations of drugs, D, which inhibit proteins in the SN. Typically, R_{SN} is measured in experimental systems as the dose dependence of output signal on drug/ligand concentration at a defined time, t, following ligand/drug application in a cellular assay. The experimental dose dependence, $R_{SN} = C_{out}$

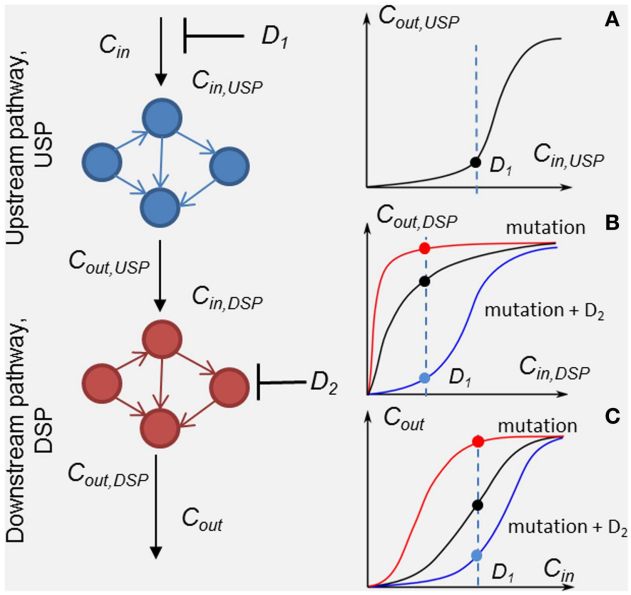

FIGURE 1 | Scheme of the method of customizing cellular signaling response by drug combination targeting upstream (drug D_1) and down-stream pathway (drug D_2). Plot sketches A, B, and C show responses, $R_{USP} = C_{out,USP}$ ($C_{in,USP}$, D_1), $R_{DSP} = C_{out,DSP}$ ($C_{in,DSP}$, D_2), and the whole SN $R_{SN} = C_{out}$ (C_{in}, D_1, D_2), respectively. Examples of the responses of unmodified DSP (black line), modified by a mutation in the DSP (red lines) and modified by the second drug D_2 (blue lines) in the presence of the mutation. Input signal C_{in} inhibited by drug D_1 is shown by the dashed lines.

(C_{in}, P_{SN}, D, t), on drug concentration allows the definition of the IC_{50} of drug D_1, which strongly depends on the target of drug D_2, the concentration of this second drug, and the mutational status of the SN determined by parameters, P_{SN}.

To analyze in detail the combination effect of two drugs at different mutations of the SN, we decompose the SN into up- and down-stream pathways that are the targets of drugs D_1 and D_2, respectively (see **Figure 1**). The response, R_{SN}, of the SN can be represented through the response R_{USP} of upstream pathway (USP) and the response, R_{DSP} of down-stream pathway (DSP) in the form, $R_{SN} = R_{DSP}(R_{USP})$, where $R_{USP} = C_{out,UPS}(C_{in}, P_{USP}, D_1, t)$ and $R_{DSP} = C_{out,DSP}(C_{in,DSP}, P_{DSP}, D_2, t)$. In this formulation, we took into account equivalences of I/O signals: $C_{in,DSP} = C_{out,USP}$ and $C_{out\_DSP} = C_{out}$ (see **Figure 1**). Theoretically, the I/O responses, R_{SN}, R_{USP}, and R_{DSP}, are readily calculated from the model of the SN. Experimentally, R_{DSP} can be extracted from two experimental dose dependencies: dose dependence of the whole SN, R_{SN}, and the receptor activation/inhibition dose dependence on activator/inhibitor concentration, R_{USP}, and represented in the form $R_{DSP} = C_{out}(R_{USP}, P_{DSP}, D_2, t)$.

Input/output response R_{DSP} determines the output signal amplitude of the DSP depending on input signals, which is controlled by the first drug D_1, the molecular parameters, P_{DSP}, and the action of the second drug D_2. Analysis of R_{DSP} allows us to study the effect of mutations, i.e., changes in kinetic properties and expression levels of proteins, P_{DSP}, on the change in R_{DSP} and how these changes determine a transition from drug sensitivity to drug resistance. Plots A, B, and C (black lines) in **Figure 1** show arbitrary responses, R_{USP}, R_{DSP}, and R_{SN}, corresponding to sensitivity of the SN to D_1. According to this type of smooth hyperbolic R_{DSP}, the output signal of the SN changes when the input signal changes under inhibitor D_1 (see black points in plots A, B, and C showing inhibited signal at drug concentration D_1 in **Figure 1**). The example of I/O response R_{DSP} modified by oncogenic mutations is shown in plot B in **Figure 1** (red line). This type of switch-like R_{DSP} corresponds to resistance of the SN to drug D_1 because the output signal of the SN does not change when the input signal changes under the action of inhibitor D_1 (see red lines and points in plots B and C in **Figure 1**). Our method provides a means to search for the protein target for drug D_2 to modify the response, R_{DSP}, in such a way as to reach two complementary effects: first, an increase in inhibition effectiveness of drug D_1 (see, e.g., R_{DSP} modified by drug D_2 in plot B, blue line in **Figure 1**); and second, to ensure robustness of this inhibition effect against different mutations in DSP (see below). One example of such a modified R_{DSP} is shown by the blue line in plot B in **Figure 1**.

To evaluate the robustness of the response R_{DSP}, we supplemented the analysis of the shape of the response curve with the SA of the DSP to both external and internal perturbations: i.e., changes in input signal and alterations of kinetic parameters and expression level of the proteins involved, P_{DSP}. We define the relative sensitivity, S_{DSP}, of the DSP to the input signal as the relative change of output signal $C_{out,DSP}$ in response to a relative change in input signal $C_{in,DSP}$, which can be written through changes in I/O responses, R_{USP} and R_{DPS}:

$$S_{DSP}(C_{in,DSP}, P_{DSP}, D_2, t) = \frac{\Delta C_{out,DSP}}{\Delta C_{in,DSP}} = \frac{\Delta R_{DSP}/R_{DSP}}{\Delta R_{USP}/R_{USP}}. \quad (1)$$

To analyze the sensitivity of the DSP response to different mutations causing changes in protein parameters (phosphorylation rate and expression level), we calculate an absolute value of relative sensitivity to changes in individual parameters p of proteins:

$$S_{DSP,p}(C_{in,DSP}, P_{DSP}, D_2, t) = \left| \frac{\Delta R_{DSP}/R_{DSP}}{\Delta p/p} \right|. \quad (2)$$

We base our local SA on the results of the application of SA to a study of the responses of various cellular signaling pathways to different mutations and drug actions. For the first time, the computational analysis of the change in sensitivity of SN depending on the protein expression level was carried out in the modeling of the apoptosis pathway (33). The SA revealed a sensitivity increase in the response of the apoptotic pathway to overexpression of Bcl-2 protein. It allowed prediction of selectivity of a Bcl-2 inhibitor for tumor cells with Bcl-2 overexpression against healthy cells with a normal level of Bcl-2. SA was first used to analyze the robustness of the MAPK cascade to oncogenic mutations in Ref. (34). The further application of the SA to an *in silico* study of the effects of the most frequent mutations in cancer (EGFR, Ras, BRaf) on the dynamics of MAPK response to receptors activation were analyzed in Refs. (35–39). An application of the SA to a study of SN response to drug action was first studied in Refs. (40, 41).

Sensitivity analysis of the I/O response allows us to: (i) determine the resistance potential of a single drug and drug combination to protein mutations in the DSP; and (ii) determine whether the DSP response to a specific drug combination is robust against various mutations in the DSP and suggest more robust combinations, which enhance the therapeutic effect in the face of mutations.

RESULTS

CHARACTERIZING SIGNALING NETWORK RESPONSE TO DIFFERENT ONCOGENIC MUTATIONS

We illustrate this method by application to the analysis of the response of PI3K/PTEN/AKT signaling to a drug combination targeting the HER2 receptor and a protein in the DSP, PI3K, which were established to be promising drug targets in both mono- and combination therapy in different cancers (1, 15, 42). The aim of the analysis of known drugs and targets is to elucidate the mechanism underlying high efficacy of their combination against different oncogenic mutations in cancer (43, 44).

The analysis is based on the kinetic model of Ras/RAF/MEK/ERK and PI3K/PTEN/AKT signaling developed in Ref. (30). This model describes the response kinetics of the SN to heregulin (HRG)-induced HER3/HER2 receptor heterodimerization and the effect of HER2 inhibitor, pertuzumab (2C4 antibody), on ERK and AKT activation in the human ovarian carcinoma cell line PE04. The scheme of the PI3K/PTEN/AKT SN that corresponds to the SN undertaken here is shown in **Figure 2**. The ordinary differential equations (ODEs) of the model and set of the model parameters are given in the Supplementary Information. In the model, we neglected other ErbB receptor heterodimers because HER2/HER3 heterodimerization activation was found to be the most mitogenic signal and induces cellular growth in the PE04 cell line (45). The model of the SN including 56 ODEs, 58 reactions, and almost 100 parameters (kinetic constants and protein concentrations) was parameterized by experimental data on the phosphorylation kinetics of HER2, ERK, AKT, and PTEN in the absence and presence of pertuzumab (30). Considering incomplete identifiability of model parameters based on the limited set of experimental data used in model calibration, we validated the model on independent experimental data on the different combination effects of PTEN, PI3K, and HER2 inhibition on the ErbB/PI3K/PTEN/AKT pathway activation (30, 32, 40, 41, 46). Model validation evidenced a good account of sensitivity of the SN to single drug action (pertuzumab) and drug combinations targeting different nodes of the SN (pertuzumab and inhibitors of PI3K, PDK1, and PTEN) (32, 40, 41, 46).

According to our method, we decomposed the ErbB/PI3K/PTEN/AKT network into two sub-networks: an upstream signaling system – the receptor system constituting HER2/HER3 receptor signaling (USP); and a down-stream pathway constituting PI3K/PTEN/AKT pathway (DSP). We used the phospho-heterodimer HER23 signal (*p*HER23) as the output signal of USP and input signal of the DSP (see **Figure 2**). The I/O response, R_{USP}, of USP was defined as the dependence of *p*HER23 concentration on the concentration of HER2 inhibitor, 2C4: $R_{HER} = p\text{HER23}(\text{HRG}, P_{USP}, 2\text{C4}, t)$. The I/O response of DSP, R_{AKT}, is the dependence of *p*AKT on concentration of phospho-heterodimer HER3/HER2, *p*HER23: $R_{AKT} = p\text{AKT}(p\text{HER23}, P_{AKT}, D_2, t)$. The input signal of PI3K/PTEN/AKT is the receptor phosphorylation signal, *p*HER23, which is changed in the calculation by varying the concentrations of the HER2 inhibitor, 2C4, in the physiological region from zero to saturated value, 1 μM (47). The dose dependence, R_{AKT}, was calculated at time $t = 30$ min after HRG and 2C4 addition that corresponds to saturation of *p*HER23 and *p*AKT signals both in modeling and experiment (30, 32).

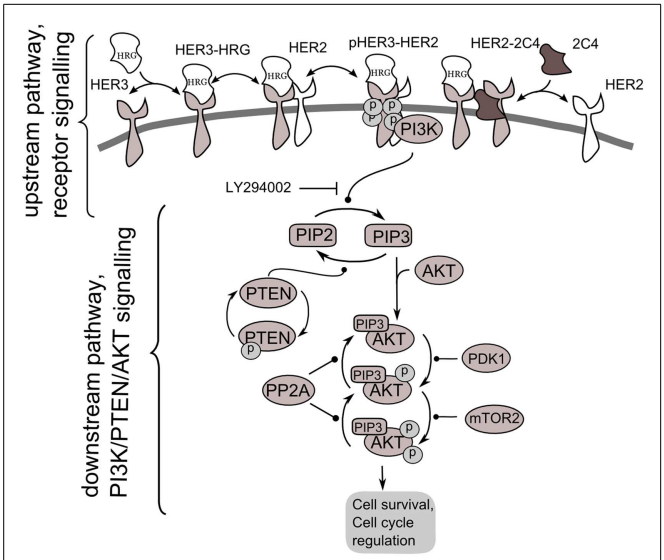

FIGURE 2 | Scheme of PI3K/PTEN/AKT signaling network activated by heterodimerization of HER2/HER3 receptors induced by heregulin (HRG) and inhibited by pertuzumab, 2C4. Decomposition of the signaling network to upstream pathway (receptor signaling pathway) and down-stream pathway (PI3K/PTEN/AKT network) is shown.

To show how the I/O response R_{AKT} depends on mutations in the PI3K/PTEN/AKT pathway, we compared the theoretical response, $R_{AKT} = p\text{AKT}(p\text{HER23}, P_{AKT}, D_2, t)$ for the unperturbed DSP (see black line in **Figure 3A**) with the response R_{AKT} modified by oncogenic mutations in the PI3K/PTEN/AKT pathway: PTEN loss (50 and 70%), variation of PI3K activity (**Figure 3B**), and 50% overexpression of AKT (see red lines in **Figures 3A–C**).

Comparing the shapes of unmodified and modified responses curves R_{AKT}, we defined sensitive and resistance modes in the DSP. In sensitive mode, an increase in *p*HER23 signal causes a gradual increase in *p*AKT signal from 0 to its saturated value (black line in **Figure 3A**). In resistance mode, the R_{AKT} curve becomes steeper and transforms to a switch-like response curve at the activation mutations (red lines in **Figures 3A,B**). The *p*AKT output signal is unresponsive to input *p*HER23 signal controlled by D_1: e.g., *p*AKT signal does not change when *p*HER23 changes in the range from 0.04 to 1 at 70% PTEN loss and activated mutation in PI3K (see red lines 3 and 4 in **Figures 3A,B**, respectively). In the case of AKT overexpression, *p*AKT signal significantly exceeds the basal activation level of *p*AKT in the range of *p*HER23 signal from 0.04 to 1 (see red line in **Figure 3C**). This lack of AKT response to input signal at

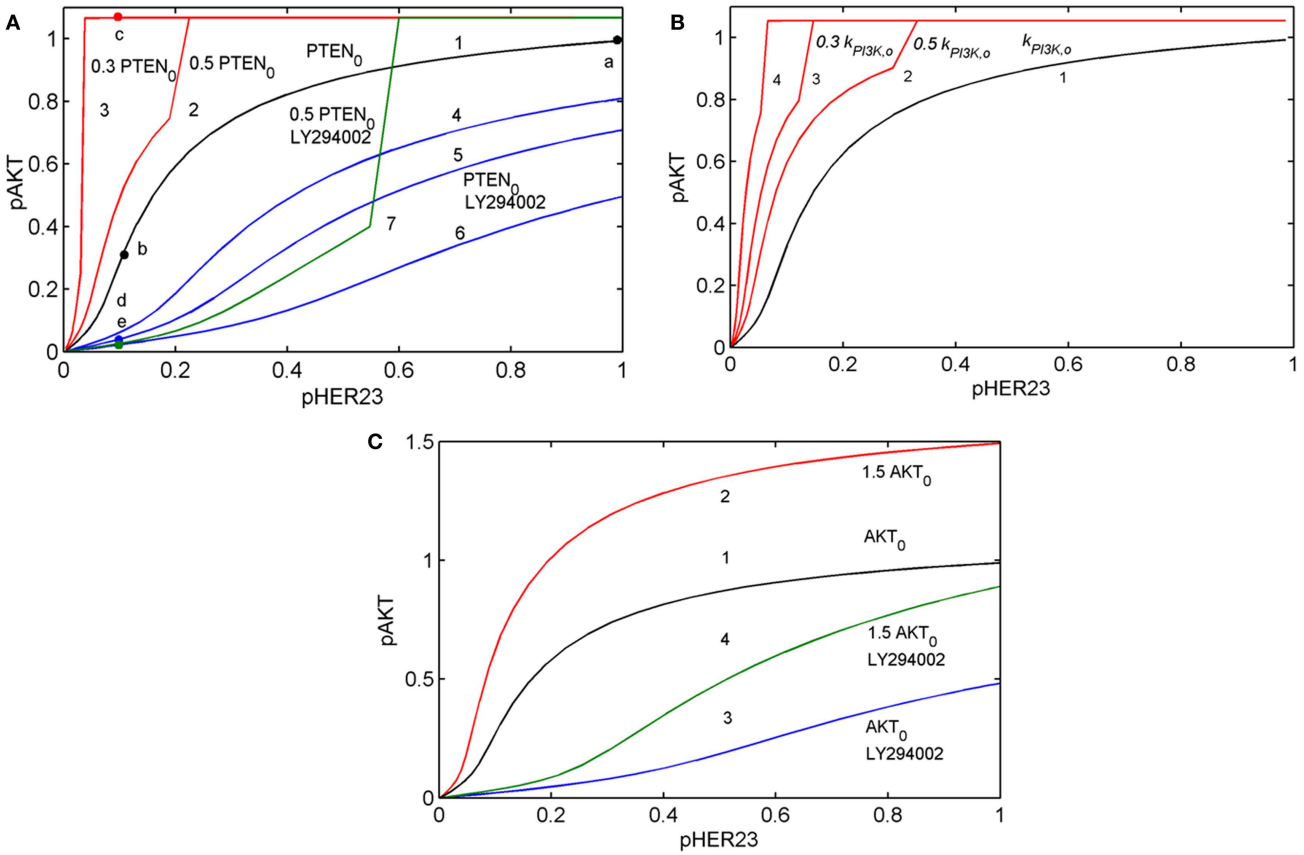

FIGURE 3 | Input/output response of the PI3K/PTEN/AKT pathway R_{AKT} to *p*HER23 input signal and its modification by activation mutations of PTEN, PI3K, and AKT proteins and inhibition of PI3K by LY294002 inhibitor. **(A)** Modification of R_{AKT} by a change of initial concentration of PTEN: response curve at the reference concentration of $PTEN_0 = 40\,\mu M$ (line 1), 50% PTEN loss (line 2), 70% PTEN loss (line 3). Modification of R_{AKT} by PI3K inhibition: 0.3 μM LY294002 (line 4), 0.5 μM LY294002 (line 5), and 1 μM LY294002 (line 6). Modification of R_{AKT} by 50% PTEN loss and inhibition of PI3K by 3 μM LY294002 (line 7). Points a, b, c, d, and e correspond to saturated *p*AKT signal (*p*HER23 = 1), its inhibition by 100 nM 2C4 (*p*HER23 = 0.1), resistance to 2C4 at 50% PTEN loss (*p*HER23 = 0.1), combined inhibition by 2C4 and LY294002 in the absence and presence of 50% PTEN loss (*p*HER23 = 0.1), respectively. **(B)** Modification of R_{AKT} by a change of PI3K activities, k_{PI3K}: reference $k_{PI3K,0}$ (line 1), 0.5 $k_{PI3K,0}$ (line 2), 0.3 $k_{PI3K,0}$ (line 3), and 0.1 $k_{PI3K,0}$ (line 4). **(C)** Modification of R_{AKT} by 50% overexpression of AKT: reference concentration AKT_0 (line 1) and 1.5 AKT_0 (line 2). Modification of R_{AKT} by 1 μM LY294002 at reference concentration of AKT_0 (line 3) and 50% overexpression of AKT (line 4).

pathway modification by mutations corresponds to the resistance of *p*AKT signal to HER2 inhibition by pertuzumab. Amongst the modifications of response R_{AKT} shown in **Figures 3A–C**, the most pronounced transition from a graded to a steep switch-like shape was observed at PTEN loss. In the model, this effect is due to the post-translational regulation of PTEN activity by its phosphorylation (see **Figure 2**) leading to the additional loss of PTEN activity (31, 48).

Analysis of R_{AKT} shows how receptor inhibition of *p*HER23 signal by D_1 transforms the functioning state of the DSP from its normal function at saturated receptor signal (*p*HER23 = 1) to non-saturated inhibited signal at low *p*HER23 <0.3 (points a and b in **Figure 3A**, respectively). When the DSP functions in non-saturation mode, the inhibited input signal differs from that in (normal) saturation mode in both a decrease in *p*AKT signal and an increase in sensitivity of the DSP to both input signal *p*HER23 and mutations causing changes in kinetic parameters and expression level of the proteins involved. To study the second effect, we analyzed the behavior of sensitivities of the DSP S_{DSP} Eq. 1 and $S_{DSP,p}$ Eq. 2 at different internal (mutations) and external (inhibition) modifications of the DSP.

The relative sensitivity of the PI3K/PTEN/AKT pathway is defined as the relative response (change) of the output signal of DSP, Δ*p*AKT, to a relative change in its input signal, Δ*p*HER23:

$$S_{AKT}(p\text{HER23}, P_{AKT}, D_2, t) = \frac{\Delta p\text{AKT}/p\text{AKT}}{\Delta p\text{HER23}/p\text{HER23}}. \quad (3)$$

Sensitivity S_{AKT} (Eq. 3) was calculated at time $t = 30$ min after HRG and 2C4 addition that corresponds to the saturation of both *p*HER23 and *p*AKT signals. Information on S_{AKT} can be obtained based on an analysis of the tangent of the response curve R_{AKT}: the steeper the response behaviors, the more sensitive the system responses to the external signal. Commonly, sensitivity increases at low input signal and this corresponds in our case to receptor signal inhibition by the first drug (see **Figure 3A**). At high

receptor signal, S_{AKT} decreases at saturated signal, pHER23 $\cong 1$. We represent the theoretical sensitivity S_{AKT} (Eq. 3) as the upper heatmap in **Figure 4** (columns 1–4) calculated at different perturbations of the DSP: (1) at the activation of DSP (pHER23 = 1); (2) at the presence of 100 nM 2C4 (pHER23 = 0.1); (3) 50% PTEN loss (pHER23 = 1); and (4) 50% PTEN loss with 100 nM 2C4 (pHER23 = 0.1). According to the shape change in response curve under these perturbations (see **Figure 3A**), the sensitivity S_{AKT} increases under 2C4 treatment in sensitive mode (compare columns 1 and 2) and decreases at 50% PTEN loss, corresponding to resistance mode (compare columns 1 with 3 and 4).

We also observed that the sensitivity of the DSP $S_{AKT,p}$ to protein parameters and expression level increases in the same region of pHER23 signal (40). Relative sensitivities $S_{AKT,p}$ (Eq. 2) of the PI3K/PTEN/AKT pathway to a specific parameter p (including kinetic parameters and expression levels of the signaling proteins) were determined as follows:

$$S_{AKT,p}(p\text{HER23}, P_{AKT}, D_2, t) = \left| \frac{\Delta p\text{AKT}/p\text{AKT}}{\Delta p/p} \right|. \quad (4)$$

To show how drug and protein mutations change sensitivity, $S_{AKT,p}$ (Eq. 4), we calculated and compared $S_{AKT,p}$ for kinetic parameters and initial concentrations of proteins involved in the PI3K/PTEN/AKT network under a normal functioning of the DPS and the mutations considered above. **Figure 4** (middle and lower heatmaps, columns 1–4) shows $S_{STS,p}$ calculated at: (1) HER23 activation by HRG (pHER23 = 1); (2) in the presence of HER2 inhibitor, 2C4, when pHER23 signal is 90% inhibited (pHER23 = 0.1); (3) at 50% PTEN loss (pHER23 = 1); and (4) at 50% PTEN loss in the presence of 2C4 (pHER23 = 0.1) (see columns 1–4 in **Figure 4**, respectively). Analysis of $S_{AKT,p}$ at normal signaling in the PI3K/PTEN/AKT network (pHER23 = 1) revealed the most sensitive modules within this network (see column 1 in **Figure 4**). The results obtained in our model are in agreement with the results of local and global sensitivity analyses of other models of the PI3K/PTEN/AKT pathway (41, 49, 50).

We observed a two-to-four orders of magnitude increase in $S_{AKT,p}$ in response to changes in the kinetic parameters and initial concentrations of the proteins at 90% inhibition of pHER23 signal by 100 nM 2C4 (compare columns 1 and 2 in **Figure 4**, lower heatmap). This increase correlates with an increase in sensitivity, S_{AKT}, at an inhibited signal of pHER23 (see upper heatmap in **Figure 4**). The increase in sensitivities S_{AKT} and $S_{STS,p}$ at low pHER23 signals corresponds to the transition of the DSP from functioning in saturated mode (pHER23 $\cong 1$) to functioning at HER2 inhibition (non-saturation). This correlation in the behavior of S_{AKT} and $S_{STS,p}$ can be readily understood: sensitivity, $S_{AKT,p}(p\text{HER23}, P_{AKT})$, to a change in P_{AKT} varies in accordance with sensitivity S_{AKT} ($pHER2$, P_{AKT}) to a change in input signal, pHER23:

$$\frac{\Delta S_{AKT}(p\text{HER23}, P_{AKT})}{\Delta p} = \frac{\Delta S_{AKT,p}(p\text{HER23}, P_{AKT})}{\Delta p\text{HER23}}, \quad (5)$$

where ΔS_{AKT} and $\Delta S_{AKT,p}$ denote sensitivity changes. Equation 5 can be derived from well-known equality of mixed derivatives

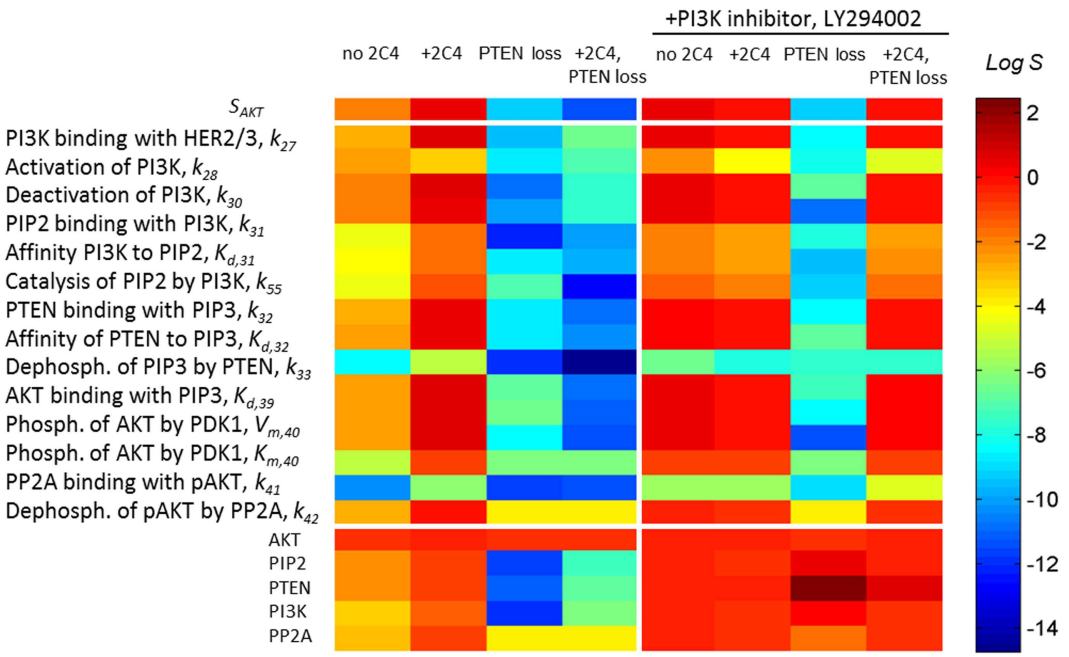

FIGURE 4 | A change in sensitivities S_{AKT} and $S_{AKT,p}$ following different perturbations in the PI3K/PTEN/AKT network. Heatmap of sensitivity S_{AKT} to pHER23 signal (upper heatmap) and sensitivity $S_{AKT,p}$ to kinetic parameters (middle heatmap) and initial protein concentrations (lower heatmap) at normal functioning of the SN (column 1), in the presence of 100 nM 2C4 (column 2), and at 50% PTEN loss in the absence (column 3) and presence of 100 nM 2C4 (column 4). Columns 5–8 show $S_{ATP,p}$ as per columns 1–4 in the presence of 3 μM LY294002. The relative change of the parameters in sensitivity analysis was 0.01% (see Eq. 4) and sensitivity values were taken at time $t = 30$ min after HRG and 2C4 addition.

for continuous function of many variables, turning to infinitesimal increments Δ in Eq. 5 – in our case for I/O response function pAKT(pHER23, P_{AKT}) depending on variables pHER23 and P_{AKT} taken at fixed time t.

We applied the results of this analysis to study the change in SN sensitivity to oncogenic mutations in the PI3K/PTEN/AKT pathway. Given an increase in sensitivity $S_{AKT,p}$ to all the parameters and expression levels of the proteins of the DSP to external perturbation (+2C4) (see column 2 in **Figure 4**, lower heatmap), we calculated sensitivity $S_{AKT,p}$ at internal perturbations: 50% PTEN loss (column 3 in **Figure 4**, lower heatmap), variation of PI3K catalytic rate, and 50% overexpression of AKT (data not shown). For all perturbations, we observed the same dramatic fall in sensitivity, $S_{STS,p}$, over almost all parameters and this drop in $S_{STS,p}$ endows the DSP with insensitivity to any further changes in kinetic parameters of proteins and their abundances. The fall of $S_{STS,p}$ correlates with the shape change of the response curve pAKT(pHER23, P_{AKT}) (red lines in **Figure 3**) and the loss of sensitivity S_{AKT} to input receptor signal pHER23 inhibited by 2C4. In contrast to a sensitive network, this insensitive DSP cannot be sensitized by drug D_1 inhibiting the input signal: the calculation of $S_{AKT,p}$ at 100 nM 2C4 showed no change in sensitivity following 50% PTEN loss (see column 4, lower heatmap in **Figure 4**). Thus, drug D_1 (2C4) acts in very different ways on unmodified and modified DSPs: it sensitizes the unmodified network (sensitive mode), but does not change sensitivity and inhibition level in the mutated DSP. Further D_1, inhibiting the input signal, shifts the DSP to a state with more sensitivity to compensation mutations, which may in turn cause the restoration of the initially activated signal and also lower network sensitivities to other external and internal perturbations.

CUSTOMIZING SIGNALING NETWORK RESPONSE THROUGH DRUG COMBINATION

Our analysis of the sensitivity of the PI3K/PTEN/AKT pathway showed that inhibition of input signal pHER23 sensitizes the response of the DSP to protein perturbations such as a change in protein expression level. Such perturbations can be also exerted by inhibition of protein phosphorylation activity. Here, we use the observed drug-induced sensitizing effect to enhance the action of the second drug D_2 targeting the DSP. Specifically, we considered PI3K inhibition by LY294002 and calculated the response of PI3K/PTEN/AKT pathway, $R_{AKT} = p$AKT(pHER23, P_{AKT}, D_2, t) to pHER23 signal at different concentrations of this inhibitor. Inhibition of PI3K modifies the response curve from hyperbolic to a smooth sigmoid form with high inhibition effect (see blue lines in **Figures 3A,C**). This modified response has a most pronounced inhibition effect of the second drug D_2 in the region of low pHER23 signal (0–0.2) where the sensitizing effect of drug D_1 is maximal (see black lines in **Figures 3A,C** and column 2 in **Figure 4**). This observation confirms that the effective and synergistic effect of the HER2 inhibitor (D_1) in combination with PI3K inhibitor (D_2) is due to a sensitizing effect of D_1 at low pHER23 signals. The key indicator of the synergistic effect of D_1 and D_2 is a change in the curvature of the response curve from convex to concave at low pHER23 signal (0.1–0.3). We assume that modification of the response curve defines the synergistic effect of HER2 and PI3K inhibitor combination (D_1 and D_2). Below, we show that this effect corresponds to a significant decrease of the IC_{50} of pertuzumab in the presence of LY294002 (see Modification of the Dose Response and its Sensitivity by Drug Combination in the Presence of Mutations).

The additional benefit of this transformation of the response curve is an insensitivity of the modified response (smooth sigmoidal shape) to oncogenic mutations in the PI3K/PTEN/AKT pathway at low pHER23 signals. To show the acquired robustness of the network in the face of these mutations, we calculated the response, $R_{AKT} = p$AKT(pHER23, P_{AKT}), at 50% PTEN loss and AKT overexpression in the presence of 3 μM LY294002 (green lines in **Figures 3A,C**). As can be seen, PTEN loss and AKT overexpression did not change significantly the inhibited signal pAKT in the range of inhibited input signal, pHER23 < 0.3 (compare state d and e in **Figure 3A** and points on lines 3 and 4 at pHER23 $= 0.1$ in **Figure 3C**, respectively). Thus, in contrast to our results on resistance to 2C4 induced by PTEN loss and AKT overexpression, these abnormalities in the context of PI3K inhibition did not result in resistance to HER2 inhibition by 2C4. Note that the advantages of that drug combination vanish at higher signals pHER23 > 0.4 where mutations and protein overexpression significantly increase pAKT signal (see lines 6 and 7 in **Figure 3A** and lines 3 and 4 in **Figure 3C**). Therefore, the response is more robust than an unmodified hyperbolic one with respect to the activation of mutations in the PI3K/PTEN/AKT pathway at inhibited pHER23 signal (pHER23 < 0.3).

This advantage of a modified smooth sigmoidal response curve was confirmed by the calculation of sensitivities, S_{AKT} and $S_{AKT,p}$, carried out in the presence of PI3K inhibitor (columns 5–8 in **Figure 4**). As can be seen, at this modification, 90% inhibition of pHER23 by pertuzumab causes a decrease in sensitivities, S_{AKT} and $S_{AKT,p}$, in contrast to their increase at the pertuzumab treatment alone (compare columns 2 and 6 in **Figure 4**). A decrease in sensitivity S_{AKT} at low pHER23 concentrations (row 1 in column 6 in **Figure 4**) confers robustness of 90% pAKT inhibition in a wider range of inhibited pHER23 signal (up to 0.3; see lines 4–6 in **Figure 3A** and lines 3 and 4 in **Figure 3C**) than in the case of hyperbolic response in the absence of the second drug. A slight decrease in $S_{AKT,p}$ in turn leads to less sensitivity to oncogenic mutations in the PI3K/PTEN/AKT pathway and suppression of drug resistance in contrast to the case of pertuzumab treatment alone. As can been seen from the I/O response curve, the robust inhibition of pAKT at low pHER23 signal is independent of the oncogenic mutations (mainly 50% PTEN loss and AKT overexpression) (compare points d and e in **Figure 3A** and points on lines 3 and 4 in **Figure 3C** at pHER23 $= 0.1$). Note this insensitivity to mutations at this drug combination vanishes at higher input signals pHER23 > 0.4, where mutations and protein overexpression significantly increase pAKT signal (see, e.g., pAKT values at pHER23 $= 0.6$ on lines 6 and 7 in **Figure 3A** and lines 3 and 4 in **Figure 3C**). Thus, the combination of HER2 inhibitor with PI3K inhibition endows the DSP with robustness against activation mutations in PI3K/PTEN/AKT pathway only at inhibited pHER23 signal in the range up to pHER23 $= 0.3$.

Note that in the model, drug D_2 causes an increase in the sensitivity $S_{AKT,p}$ to the protein parameters in comparison with the

unperturbed SN that can sensitize the DSP to compensatory mutations (see columns 1 and 5 in **Figure 4**). This discrepancy may be due to the fact that in the calculation we compared the sensitivity at inhibited (pHER23 = 0.1) and saturated (pHER23 = 1) pHER23 signals (points d and a in **Figure 3A**, respectively). Here we assumed that the activation growth factor signal (1 nM HRG) is saturated (point a in **Figure 3A**). However, if we assume ligand concentration to be lower than the saturation level (e.g., 0.6–0.8) (51) and compare the sensitivities at inhibited and this pHER23 signal, we find that drug D_2 does not change the sensitivity in comparison with the unperturbed DSP (data not shown).

MODIFICATION OF THE DOSE RESPONSE AND ITS SENSITIVITY BY DRUG COMBINATION IN THE PRESENCE OF MUTATIONS

To show how the modifications of I/O response of the AKT subsystem, R_{AKT}, effect on the dose dependence of the whole ErbB/PI3K/PTEN/AKT network for drug D_1, we calculated pAKT and $S_{AKT,p}$ dose dependencies for pertuzumab at different perturbations of the PI3K/PTEN/AKT subsystem discussed above (see solid and dashed lines in **Figure 5**, respectively). Specifically, we calculated sensitivity $S_{AKT,k31}$ to the rate constant of the reaction of PI3K binding with PIP2, k_{31} (see **Figure 2** and model description in Supplementary Information). The calculation for other parameters p was shown to effect the same results (as guaranteed by the general behavior of $S_{AKT,p}$ for all p, according to the discussion of Eq. 5). In the case of an unperturbed DSP, sensitivity $S_{AKT,k31}$ increases by approximately 20 times (relative to its level in the absence of pertuzumab) and has a peak at 80% inhibition of pAKT (IC_{80} for pertuzumab) (see black circle and square on the dashed and solid black lines at 100 nM pertuzumab in **Figure 5**, respectively). Key features of the dose dependence $S_{AKT,k31}$ calculated with the unperturbed PI3K/AKT module are the presence of maximum in the range of IC_{80}, a non-zero limit at high drug concentrations, and a limiting value at low drug concentration, which corresponds to the sensitivity of the signaling system in the absence of the drug (see heatmap in **Figure 4**). The detailed analysis of these features is given in Supplementary Information (see Figures S1 and S2 in Supplementary Material).

The calculation of the pAKT dose dependencies for pertuzumab in the presence of PI3K inhibitor showed the shift of the dose dependence curve to lower drug concentrations and a decrease of IC_{50} for pertuzumab from 30 nM to 1 nM at an increase in LY294002 concentration up to 3 μM (see solid blue line in **Figure 5** and Figure S2 in Supplementary Material). In contrast to dose dependence, calculation of relative sensitivity $S_{AKT,k31}$ revealed a complex behavior that only moves to low pertuzumab concentrations at increasing LY294002 concentration: the maximum near IC_{80} disappears and sensitivity behavior becomes almost monotonically decreasing with a slight minimum (see Figure S2A in Supplementary Material). We assume that a shape change of $S_{AKT,k31}$ at the shift of the dose dependence is the manifestation of transformation of the DSP response curve (**Figure 3A**). Sensitivity $S_{AKT,k31}$ at 3 μM LY294002 does not have maximum at IC_{80} and decreases from its initial value at low 2C4 concentration to approximately its limit value at high pertuzumab concentration (see blue circle and square on the dashed and solid blue lines, respectively at 6 nM pertuzumab in **Figure 5**). This dose dependence corresponds to the modified response curve (line 6 in **Figure 3A**). We predict a significant decrease of IC_{50} for pertuzumab in the presence of 3 μM LY294002 due to the synergistic combination of these two drugs and explain this effect by modification of the response of PI3K/PTEN/AKT pathway at low input signals (line 6 in **Figure 3A**).

Calculation of absolute sensitivity, $S_{AKT,k31}$ (Eq. S2.1 in Supplementary Material), showed that it possesses similar features except for a zero limiting value at high drug concentration (see Figure S2B in Supplementary Material). Similarly, at 3 μM LY294002, absolute sensitivity loses its maximum in the range of IC_{80} and

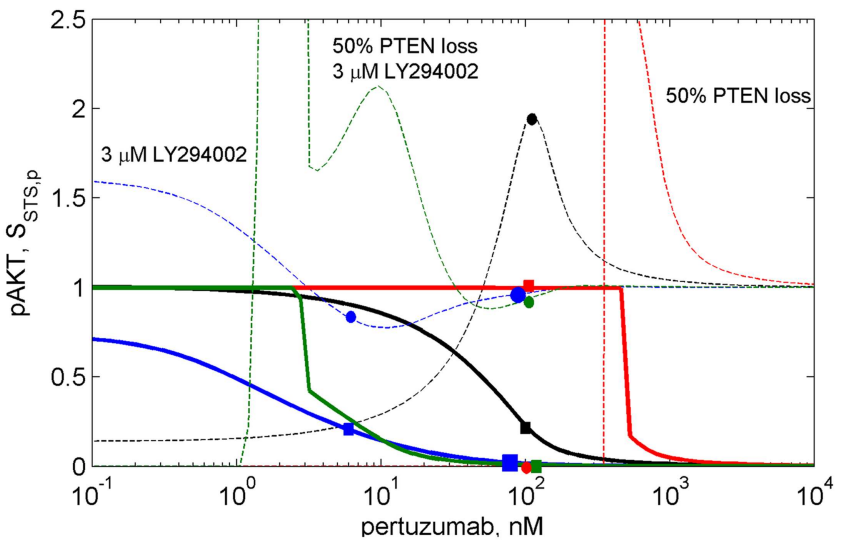

FIGURE 5 | The dose dependences of *p*AKT inhibition (solid lines) and sensitivity $S_{AKT,k31}$(2C4,p) of the PI3K/PTEN/AKT network to the rate constant of the reaction of PI3K binding with PIP2, k_{31} (dash lines) at different drug combinations. The dose dependencies of inhibition and sensitivity on pertuzumab concentration (black lines), at 50% PTEN loss (red lines), at 3 μM LY294002 (blue lines), at 50% PTEN loss and 3 μM LY294002 (green lines).

monotonically decreases when drug concentration increases. We consider this transformation of sensitivity behavior as the results of modification of the DSP dose response from hyperbolic to smooth sigmoid shape (**Figure 3**). This transformation, and the range of second drug concentration where it occurs, can be used in optimization of drug composition to inhibit signaling, so avoiding a significant increase in sensitivity of the SNs to mutations. Below we consider one such optimization.

Transformation of the response of the DSP module leads to *p*AKT inhibition by two drugs to exceed 80% in the wide range of LY294002 concentrations (0.3–3 μM) at a significantly lower concentration of pertuzumab (6 nM) in comparison with *p*AKT inhibition by pertuzumab alone, 100 nM (see **Figure 5**; Figure S2 in Supplementary Material). This allows optimization of drug concentrations with respect to both inhibition of *p*AKT signal and sensitivity of the SN to mutations. Since these two characteristics are interlinked and change correlatively in the same region of the response curve (**Figure 3A**) and dose dependence (**Figure 5**), the problem of trade-off between them arises. It is possible to disentangle inhibition and sensitivity characteristics from each other by optimal manipulation of two drugs in the concentration range discussed above. One can establish two optimized conditions of drug combination action with high inhibition and low sensitivity. The first optimum condition is to decrease 2C4 concentration to maintain 80% inhibition of *p*AKT ($IC_{80} = 6$ nM) with low sensitivity at 2–3 μM LY294002 (see blue square and circle on solid and dashed blue lines respectively in **Figure 5** and lines 5 and 6 in Figure S2 in Supplementary Material). The second optimum is full inhibition of *p*AKT with 100 nM pertuzumab and a minimal increase (six times) of sensitivity at 3 μM LY294002 (see large blue square and circle on solid and dashed blue lines respectively in **Figure 5**).

To demonstrate the sensitivity to resistance transition in response to HER2 inhibition at PTEN loss, we calculated the dose dependences of *p*AKT and $S_{AKT,p}$ at 50% PTEN loss (see red solid and dashed lines in **Figure 5** and corresponding I/O response of the *p*AKT, line 2 in **Figure 3A**). PTEN loss leads to a 10-fold shift of *p*AKT dependence to a higher pertuzumab concentration, so causing resistance to HER2 inhibition in the range of physiological pertuzumab concentration (100 nM). In this region of drug concentration, both inhibition of *p*AKT and sensitivity $S_{AKT,p}$ are approximately zero (see red square and circle on solid and dashed red lines in **Figure 5**, respectively). Sensitivity to HER2 inhibition is restored through modification of the network by PI3K inhibitor, LY294002. This modification causes approximately full inhibition of *p*AKT (green square on green solid line in **Figure 5**) while sensitivity $S_{AKT,p}$ (green circle on green dashed line in **Figure 5**) is at approximately the same level as for normal PTEN concentration.

Joint analysis of the I/O response of the PI3K/PTEN/AKT pathway and *p*AKT dose dependence for pertuzumab (drug D_1) revealed a trade-off between inhibition and sensitivity, which can be formulated as follows. If the concentration of drug D_1 is high (low *p*HER23 signal), *p*AKT inhibition is strong and sensitivity to protein parameters is low: this is of a benefit with respect to signal inhibition and suppression of compensatory mutations. If the concentration of D_1 is low (high *p*HER23 signal), *p*AKT concentration increases while sensitivity decreases, which gives advantages with respect to mutation suppression but leads to a decrease in inhibition effect of the drug, D_1. If the concentration of drug D_1 is in the region of its IC_{50}, sensitivity increases which affords the use of the second drug more effectively (drug-sensitizing effect), and a disadvantage with respect to increasing sensitivity of the SN to compensation mutations. This analysis showed the inhibition-sensitivity trade-off decision can be optimized by customizing the I/O response of the SN through manipulation of the concentrations of the two drugs. Optimization of drug concentration allows the separation of the regions with high inhibition and high sensitivity, which overlap each other in single drug treatment.

DISCUSSION

We have shown that the sensitivity of the SN to drug action is attended by an increase in sensitivity $S_{DSP,p}$ of the DSP to the kinetic parameters and expression levels of the proteins involved in this pathway. As a result, the DSP is sensitized by drug action and may be more fragile with respect to mutations, which change protein kinetic properties (catalytic or/and dissociation constants) and their expression level (overexpression or suppression of gene expression). In particular, an increasing sensitivity may result in an adverse effect of inhibitor action since a high sensitivity endows the SN with fragility with respect to mutations that can compensate for the intended inhibitor effect by restoring a high output signal as well as initial low sensitivity of the SN to external perturbations. It follows that increasing SN sensitivity can be one of the causes of resistance potential to drug action. We suggest that this effect should be taken into account at drug target validation, and the drug resistance potential linked with this sensitizing effect should be evaluated in drug combination co-development. We present a method to design drug combination strategies that modify the I/O response of the SN to minimize any drug-sensitizing effect, enhance robustness of drug inhibition effect, and improve drug resistance potential by vertically targeting the SN.

The method is based on the modularity approach to analyzing the efficacy of combination therapy developed by Fitzgerald et al. (52). According to this approach, we divide the signaling system into up- and down-stream pathways, which are the targets of the first and second drugs, respectively (see scheme in **Figure 1**). In this work, we focused on the I/O response characteristics of the down-stream pathways, DSP, which is a signal transduction module in the whole signaling pathway. As shown in Ref. (52), the effect of drug combination significantly depends on the I/O response properties of the receptor systems, and mainly receptor expression level, that shifts the IC_{50} concentration of drug, D_1. To exclude the effects determined by the I/O response properties of the receptor system, we considered only the output signal of the USP which is varied from zero to a saturated level. In this modularity approach, the effect of drug D_1 defines only the input signal for the I/O response curve of the DSP (see **Figures 1** and **3A**). We expanded this approach developed by Fitzgerald et al. (52) to investigate the modification of the I/O response of the down-stream pathways as a result of external and internal perturbations such as inhibition of signaling proteins and protein mutations. The analysis of the responsiveness of the whole system to the first drug was shown to depend significantly on the I/O response characteristic of the down-stream signaling module. We suggest that the response curve, R_{DSP}, can be considered as

a biomarker (characteristic signature) of a particular cancer signaling pathways (e.g., MAPK, PI3K/AKT, Wnt, and others (53) in specific cancer cell lines. To illustrate this, we have extracted the I/O response R_{DSP} of the PI3K/PTEN/AKT and MAPK pathways for different cancer cell lines using experimental data on the dose dependence of receptor activation (EGFR and PDGFR) and output signal (*p*AKT and *p*ERK) on ligand concentrations (EGF and PDGF) (49, 54, 55). The characteristic I/O response curves obtained for different activating input signals in various cell lines are shown in **Figure 6**. The experimental data on I/O responses were fitted by the Hill function with a Hill constant, *n*, which characterizes that response as switch-like (higher *n*) or more graded (lower *n*). As shown, AKT responses are more varied compared with the more conserved set of ERK responses that are typically switch-like ($n = 2.65–12.8$). *p*AKT responses range from switch-like for HBL and AU565 cells (**Figures 6A,B**) to graded hyperbolic for T47 and PE04 cells (**Figures 6C,E,F**) and smooth sigmoidal for MCF7 cells (**Figure 6D**) responses. We assume that such variety in the responses of the PI3K/PTEN/AKT pathway results from a variation in the internal parameters of the networks caused by mutations and different expression levels of signaling proteins. We suggest that I/O responses can be considered as a biomarker of mutation and protein expression status of specific cancer cells.

The *in silico* analysis of the I/O response of the PI3K/PTEN/AKT pathway confirmed that the SN can possess a smooth hyperbolic response, which corresponds to the sensitivity mode in signal transduction, and this response can be transformed into a switch-like response by changing protein parameters, which corresponds to activating mutations of the proteins involved (PTEN, PI3K, and AKT). This transformation relates to the transition from sensitivity to resistance of the SN to drug inhibiting receptor signals (32). Joint analysis of the I/O response of the PI3K/PTEN/AKT pathway and its sensitivity showed that these two key characteristics of the cellular SN are interconnected and they both significantly depend on the protein parameters and their expression levels. We observed from *in silico* experiments that perturbations of protein parameters corresponding either to 50% decreases in PTEN concentration (PTEN loss), PI3K activation mutation, or AKT overexpression significantly changes in the I/O response of the PI3K/PTEN/AKT pathway and its sensitivity. The transformation of the I/O response from smooth hyperbolic to switch-like at PTEN loss significantly endows the SN with hypersensitivity (56) that is assumed to be typical for the signaling cascade in cells at decreasing phosphatase activity (38). The steep switch response observed at PTEN loss is assumed to be due to a post-translational regulation of PTEN activity by its phosphorylation leading to the additional loss of PTEN activity (31, 48) considered in our model.

We showed that PTEN loss, or *PIK3CA* activation mutation, AKT and PI3K overexpression (40) decreased sensitivity $S_{AKT,p}$ by four to six orders of magnitude for all protein parameters and this corresponds to the transformation of the I/O response from smooth hyperbolic to switch-like. At this mutation, the PI3K/PTEN/AKT pathway acquires insensitivity to further

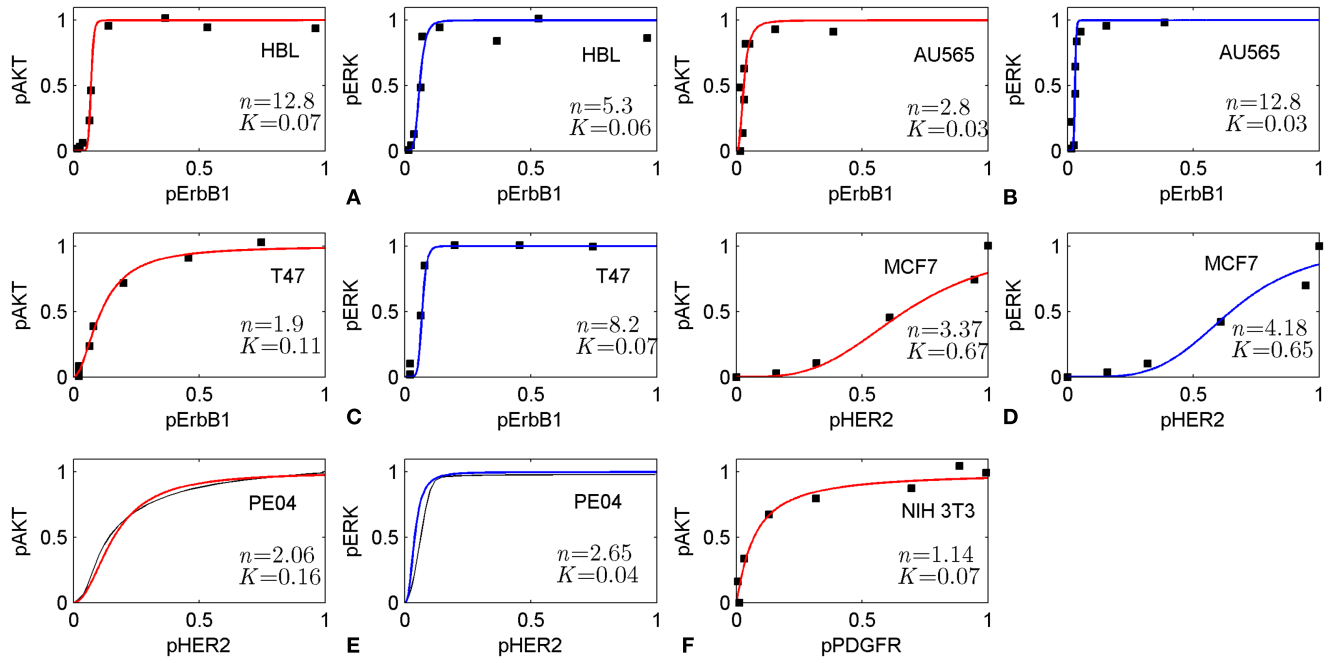

FIGURE 6 | Input/output responses of PI3K/AKT and MAPK pathways to the activation of growth factor receptors in different cells. The dependences of *p*AKT and *p*ERK on the activation level of ErbB1 receptors in HBL100 **(A)**, AU565 **(B)**, T47 **(C)** cells; on the level of HER2 receptors in MCF7 **(D)** and PE04 **(E)** cells; and platelet-derived growth factor receptor (PDGF) in NIH 3T3 fibroblasts **(F)**. Data on I/O response were extracted from experimental dose dependences [**(A–C)** (49), **(D)** (54), and **(F)** (55)] and normalized at maximal values of input/output signals. *p*AKT, *p*ERK dose dependencies for PE04 cells **(E)** are theoretical data (40) (black lines). Experimental data were fitted by Hill function: $y = x^n/(x^n + K^n)$ with Hill constant, *n*, and half-maximum constant, *K*, which are given in Figures. Hill function is depicted by red lines for *p*AKT and blue lines for *p*ERK. Experimental data were kindly allowed to be used by PK Sorger **(A–C)**, M Hatakeyama **(D)**, and JM Haugh **(E)**.

perturbation of the SN: it becomes robust in the face of both external perturbations (inhibition of input signal) and internal aberrations (mutations). PTEN loss leads to resistance to drug action (resistance to HER2 inhibition) and causes robust activation of the AKT output signal (57). Note that the observed decrease in sensitivity of the SN at either PTEN loss or PI3K aberration suggests that PTEN loss and *PIK3CA* mutation are mutually exclusive. Clinical study of oncogenic mutations in the PI3K/PTEN/AKT pathway reported both the data on mutually exclusive of PTEN loss and *PIK3CA* mutation in human breast (58) and gastric (59) carcinomas and frequently concordant in breast cancer (43, 44). As both mutations activate AKT and each decreases sensitivity of the DSP to another mutation, our finding suggests either redundancy in mutations, the need for two alterations in the single module to activate AKT, or that PTEN loss and *PIK3CA* mutation contribute differently to carcinogenesis (60).

Combining analysis of the I/O response with SA, we showed that a hyperbolic response in sensitivity mode is robust at high input (saturated receptor signals) and loses its robustness against activation mutations of the proteins at low *p*HER23 signals. Importantly, the I/O response can be modified through drug combination action, here through customization of the I/O response of the DSP by drug D_2 to reach high robustness of the inhibition response to drug D_1 against oncogenic protein mutations in the pathway, leading to the transformation of a hyperbolic response to a graded sigmoidal response curve (see blue lines in **Figure 3A**). The first benefit of that modification is an effective inhibition of output signal, *p*AKT. The most pronounced inhibition effect of the second drug is at low *p*HER23 region (0–0.2), where the sensitizing effect by the first drug, pertuzumab, is at a maximum. We exploit a synergistic interaction between these two drugs, specifically the sensitizing effect of one drug on the action of another. A second benefit of that modification is a decrease in the sensitivity of this response in the range of low *p*HER23 signal (0–0.2) (see blue lines in **Figure 3A**). We hypothesized that the modified response is more robust against oncogenic mutation than the hyperbolic type of the response. To check this, we carried out robustness screening of this response with respect to the following aberrations in the PI3K/PTEN/AKT pathway: PTEN loss, activation mutation of PI3K, and overexpression of AKT. In all these cases we observed effective inhibition of *p*AKT and so a high degree of robustness in the modified response to common oncogenic mutations in the PI3K/PTEN/AKT pathway (see green lines in **Figures 3A,C**). We showed that a hyperbolic response is more adapted to signal discrimination and signal transaction while a modified graded sigmoidal response close to linear response is more adapted to therapeutic inhibition of signaling pathway activated by oncogenic mutations.

Note, a similar transformation from hyperbole to switch-like response was observed *in silico* and *in vitro* experiments in Raf/MEK/ERK pathway as a result of deletion of negative feedbacks from ppERK to Son of Sevenless protein (SOS) and Raf (26). This finding and other theoretical and experimental studies show that multiple feedback and gene regulation can significantly control drug efficacy (4, 5, 52). An extension of the modularity approach used in our work should be performed to explore the consequences of feedback and gene regulation networks, which control the robustness of SNs in normal and malignant conditions (61, 62). For example, to develop more reliable model of SN response to drug combination, it is necessary to take into account the negative feedback in the PI3K/PTEN/AKT pathway which includes phosphorylation (inactivation) of GSK3β by *p*AKT which phosphorylates (inactivates) PTEN, and therefore increases AKT phosphorylation (31).

As discussed in the Section "Introduction," many experiments and clinical trials confirm the suppression of drug resistance and the robustness response of drug combinations acting on the vertical signaling pathways in different cancers. For example, complementary inhibition of the vertical targets in the PI3K/PTEN/AKT/mTOR pathway restores the inhibition effect of trastuzumab and pertuzumab (15, 17). Moreover, the experimental study of the combination of PI3K inhibition (GDC-0941) with HER2 inhibitors (trastuzumab, pertuzumab) showed that the high efficacy of this combination therapy is a general effect for different breast cancer cell lines despite the fact that different cell lines have different susceptibilities/resistance to each drug separately (42).

Our *in silico* experiments indicate that anti-cancer response robustness to drug action emerges due to a smooth sigmoid response of the SN when modified by drug combination. Experimentally, the robustness of the modified response to a change in expression level of signaling proteins can be measured in RNA interference (RNAi) screening with respect to repression of protein expression. RNAi screening has been applied successfully to a study of resistance mechanisms and development of effective combination therapy by determining drug targets (60, 63), and allows estimation of the robustness of the therapeutic response to the perturbations in expression of the proteins (isomers or catalytic/regulatory protein subunits) surrounding drug targets in a SN (both vertical and horizontal targeted pathways). Robustness screening with respect to overexpression of the proteins surrounding targeted proteins can be measured using isogenic cell clones customized to genomic status involved in screening (64). Most critical experimental validations are assumed to be carried out with heterogeneous tumor samples. Here we suggest that designing into a combination therapy regime, an unchanged landscape of sensitivity across a mutation spectrum, such as that shown in heatmap in **Figure 4** (columns 5, 6, and 8), may slow down the evolution of subpopulations of cancer cells under a selection pressure of drug therapy and suppresses the development of drug-resistant clones (28, 65). Further, effecting this slowing down in resistance evolution may require a longer-term engineered drug combination regime that accounts for a drift in cell signaling behavior over many cellular generations.

The method of investigation of the I/O response of the SN modules developed here can be considered as a supplementary tool for the analysis of the dose dependence for drug D_1 and the mechanism of IC_{50} changes at oncogenic mutations and combination therapy. To show the link between response dynamics properties of the PI3K/PTEN/AKT pathway and the dose response of the whole SN, we calculated the dose dependence of *p*AKT on concentration of pertuzumab (drug D_1): $R_{SN} = C_{out}(C_{in}, D, P_{SN}, t)$, and showed how the modification of the DSP response by mutations and drugs changes IC_{50} for pertuzumab (**Figure 5**). In the calculation, we observed a decrease of IC_{50} for pertuzumab in the

presence of PI3K inhibitor, LY294002, suggesting lower concentrations to be effective. These results supplement the analysis of the drug-induced shift in dose dependence and the synergetic effect of two drugs targeting vertical pathways discussed by Fitzgerald et al. (52). Additionally, we showed that drug-induced sensitivity to the second drug modify both response curve and dose dependence for the first drug that significantly enhances synergetic effect of two drugs in combination.

We showed that the sensitivity of the DSP without customization increases significantly in the range of IC_{80} of pertuzumab (see solid and dashed black lines in **Figure 5**). The simplest way to reach the goal of high inhibition and low sensitivity is to increase the drug concentration and go far beyond the range of maximal sensitivity ($>1\ \mu$M pertuzumab, **Figure 5**). In practice, this is often not a realistic solution for toxicity reasons, although there is evidence that drug concentrations that are twice the IC_{50} can suppress acquired resistance due to mutations following drug therapy (29). A more realistic approach is to customize the network response to inhibition through combination therapy, manipulating the inhibition and sensitivity dose dependencies through the action of the second drug, which modifies the I/O response of the DSP. Additionally, our calculation showed that a significantly lower IC_{50} for pertuzumab in combination with the PI3K inhibitor can be a basis for decreasing a dose of drug D_1 to reduce its toxicity.

We applied the method to two well-known targets and drugs and investigated the mechanism of their effectiveness. We demonstrated that the drug-induced sensitivity of the DSP to changes in protein parameters and their expression levels has the potential to sensitize this pathway to mutations that compensate for drug action, i.e., low output signal at inhibited input signal. Moreover, such mutations can effect a decrease in sensitivity of the DSP to further external and internal perturbations leading to an increase in DSP robustness. It is thus possible that drug action may significantly perturb DSP functioning, stimulating the activation of compensatory response mechanisms in the cell.

We then demonstrated a method of minimizing the drug-sensitizing effect while maintaining the intended effect of drug action by customizing the I/O response of the DSP response. The goal of this customization was to reach a high inhibition of input signal without sensitizing the SN to mutations leading to an increased potential for resistance, specifically PTEN loss and AKT overexpression. Our focus was the modification of the I/O response of the PI3K/PTEN/AKT pathway to inhibition of pHER2 by pertuzumab.

These results suggest that the I/O response of signaling modules can be used as biomarker to select advantaged drug targets for mono- and combination therapy. To illustrate this concept, we considered different strategies of drug selection depending on the response of the PI3K/PTEN/AKT signaling module of the different cells represented in **Figure 6**. We assume that in the case of switch-like response with low threshold of activation (low parameter K) such as represented in **Figure 6A** (HBL cells) and **Figure 6B** (AU565 cells), a rational strategy is to use drug combination in which one drug targets the DSP and modifies the response curve to more graded or sigmoid form while another drug in combination inhibits input and output signals according to that modified response curve. In the case of responses of smooth hyperbolic type like represented in **Figure 6C** (T47), **Figure 6E** (PE04), and **Figure 6F** (NIH 3T3), the drug targeting USP effectively inhibits *p*AKT in the 80–90% range of inhibited receptor signal but sensitizes down-stream module to compensation mutations. To avoid the latter effect, we suggest modifying the response of the down-stream module to a smooth sigmoid curve by a second drug targeting the DSP. The I/O smooth sigmoid response of the type given in **Figure 6D** (MCF7) is assumed to endow the signaling module with high sensitivity to the drug inhibiting its input signal and low sensitivity to compensatory mutations. In this case, inhibition of the USP by one drug is effective with respect to both signal inhibition and sensitivity suppression. The use of the second drug targeting down-stream pathway is redundant from the viewpoint of the response modification and toxicity. These analyses indicate the strategy of drug–diagnostic co-development (2, 66) when drug therapies (mono- or combination) are selected based on the integrative biomarker corresponding to the I/O response of signaling modules. Stratification of patients according to different I/O response curves can help identify patients who are most likely to benefit from selected therapy. Constant monitoring of the transformation of the I/O response curve during treatment can indicate at compensatory gene regulation (12, 45, 67, 68) and acquired mutations as a result of the selected therapy and so can serve as a guide for changing therapeutic regime. The obtained results showed that it is desirable to introduce an additional characteristic of drugs to support this strategy – drug-induced sensitivity of the SN or resistance potential of drugs, which significantly depends on the I/O response of targeted SN.

The joint analysis, the I/O response of the PI3K/PTEN/AKT pathway, and *p*AKT dose response for pertuzumab revealed the challenge of the trade-off between high sensitivity to drug action and oncogenic mutations. The proposed method of modification of the I/O response of the PI3K/PTEN/AKT pathway and manipulating separately by the inhibition and sensitivity dose dependencies through action of a second drug resolved this problem partially. Despite PI3K inhibition causing the loss of relative sensitivity at different perturbations (pHER2 inhibition and the different mutations in PI3K/PTEN/AKT pathway), it increased the overall level of sensitivity of the DSP to protein parameters that is assumed to sensitize the SN to the compensation mutations (compare columns 1 and 5 in **Figure 4**). To further optimize this trade-off at the combination design stage, we propose that other means of customizing the I/O response to improve drug resistance potential are needed, e.g., by using the methods and results of synthetic biology in signaling pathway engineering to customize their I/O responses (69–71). For example, using some modulators it is possible to alter/reshape dose dependence from a graded to a sharply sensitive, switch-like response and a time dependence from sustained to pulse or delayed responses in MAPK pathway signaling (71). It has been shown that modification of cellular response obtained by genetic engineering can also be reached through drug action. For example, cellular responses were engineered by dynamic rewiring of SN topology (22, 72) and controlling negative feedback circuits in SN by drug combination (26). Ultimately the application of this arsenal of engineering methods in network and synthetic biology, underpinned by integrative systems biology, can be a powerful tool for adaptation of signaling response to effective combination therapy and rational drug combinations co-development.

ACKNOWLEDGMENTS

This work was supported by grants from Breakthrough Breast Cancer and Scottish Funding Council (SRDG), and personal support to Alexey Goltsov from Scottish Informatics and Computer Science Alliance (SICSA) and to James Bown from The Northwood Trust. We also acknowledge the Reviewers for detailed analysis of the method proposed and valuable comments.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fonc.2014.00013/abstract

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 12 October 2013; accepted: 20 January 2014; published online: 05 February 2014.

*Citation: Goltsov A, Langdon SP, Goltsov G, Harrison DJ and Bown J (2014) Customizing the therapeutic response of signaling networks to promote antitumor responses by drug combinations. Front. Oncol. **4**:13. doi: 10.3389/fonc.2014.00013*

This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology.

frontiers in ONCOLOGY

ORIGINAL RESEARCH ARTICLE
published: 15 August 2014
doi: 10.3389/fonc.2014.00207

miR-21 expression in cancer cells may not predict resistance to adjuvant trastuzumab in primary breast cancer

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Trastuzumab is established as standard care for patients with HER2-positive breast cancer both in the adjuvant and metastatic setting. However, 50% of the patients do not respond to the trastuzumab therapy, and therefore new predictive biomarkers are highly warranted. MicroRNAs (miRs) constitute a new group of biomarkers and their cellular expression can be determined in tumor samples by *in situ* hybridization (ISH) analysis. miR-21 is highly prevalent and up-regulated in breast cancer and has been linked to drug resistance in clinical and *in vitro* settings. To determine expression patterns of miR-21 in high-grade breast cancers, we examined miR-21 expression in 22 HER2-positive tumors and 15 HER2-negative high-grade tumors by ISH. The histological examination indicated that patient samples could be divided into three major expression patterns: miR-21 predominantly in tumor stroma, predominantly in cancer cells, or in both stromal and cancer cells. There was no obvious difference between the HER2-positive and HER2-negative tumors in terms of the miR-21 expression patterns and intensities. To explore the possibility that miR-21 expression levels and/or cellular localization could predict resistance to adjuvant trastuzumab in HER2-positive breast cancer patients, we analyzed additional 16 HER2-positive tumors from patients who were treated with trastuzumab in the adjuvant setting. Eight of the 16 patients showed clinical recurrence and were considered resistant. Examination of the miR-21 expression patterns and intensities revealed no association between the miR-21 scores in the cancer cell population ($p = 0.69$) or the stromal cells population ($p = 0.13$) and recurrent disease after adjuvant trastuzumab. Thus, our findings show that elevated miR-21 expression does not predict resistance to adjuvant trastuzumab.

Keywords: biomarker, breast cancer, HER2, miR-21, prediction, response, trastuzumab

INTRODUCTION

The human epidermal growth factor receptor-2 (HER2, NEU, and c-ERBB-2) is a cell surface receptor tyrosine kinase that is strongly up-regulated in more than 15% of all breast cancers (1), and breast cancer patients with HER2 overexpressing tumors have poor prognosis (2, 3). HER2-directed therapy using the humanized monoclonal antibody, trastuzumab in combination with conventional chemotherapy, improves overall survival in patients with HER2-positive breast cancers (4). However, only half of the HER2-positive breast cancer patients respond to the HER2-directed therapy (5). The lack of therapeutic efficacy and high cost of the therapeutic agent urges identification and development of predictive markers as companion diagnostics. So far, no clinically validated predictive markers for response to trastuzumab have been reported.

MicroRNAs (miRs) are short non-coding RNAs that regulate protein synthesis at the post-transcriptional level by binding to the 3'UTR of mRNAs causing mRNA destabilization or degradation. miRs can be measured in tissues and body fluids by *in situ* hybridization (ISH), qPCR, and microarray or high-throughput sequencing, and as such, miRs constitute a relatively novel group of biomarkers. One of the most abundant miRs in solid tumors is miR-21. miR-21 expression is highly increased in malignant tumors, including breast, colon, lung, and brain cancer (6, 7), and high expression levels are associated with poor prognosis (8–11). Solid tumors, including breast cancers, are complex tissues consisting of the malignant epithelial cells and a surrounding stroma consisting of fibroblasts, inflammatory cells, and vascular cells. ISH studies of tissue from breast, colon, brain, pancreas, and esophagus cancer (10–14) have shown that miR-21 is predominantly expressed in the stromal cells; however, subpopulations of miR-21 positive cancer cells are also reported (10, 11, 15).

Some miRs are found to be indicators of drug resistance and some even to confer drug resistance to a variety of cancer drugs (16–18). miR-21 is one the most studied and has been found to confer drug resistance to trastuzumab (19), 5-fluorouracil (20, 21), doxorubicin (22, 23), cisplatin (24, 25), and paclitaxel (26). Gong et al. (19) reported that high miR-21 levels in breast cancer

biopsies before and after neoadjuvant trastuzumab were associated with trastuzumab resistance, and that blocking the action of miR-21 re-sensitized resistant breast cancer cell lines to trastuzumab (19). The authors suggested that miR-21 may function through the tumor suppressor phosphatase and tensin homolog (PTEN), a well-described miR-21 target protein (27–29), which has been reported to be a potential predictor for trastuzumab resistance (30). Thus, miR-21 may play an important role in multi-drug resistance mechanisms.

Recent studies have indicated that drug resistance in cancer therapy is not only related to the malignant cancer cells, but also to cells in the stromal compartment (31–34). For example, in the study by Alkhateeb et al. (34), high levels of inflammation markers measured in serum from HER2-positive breast cancer patients were associated with poor response to trastuzumab-containing therapy. Thus, molecular biomarkers derived from the breast cancer stroma should also be considered in the search for novel predictive biomarkers.

In this paper, we addressed whether miR-21 ISH analysis in primary breast cancers can help to predict trastuzumab resistance in HER2-positive breast cancer patients treated with trastuzumab in the adjuvant setting, and whether the miR-21 expression pattern correlated with HER2 status or other known clinical markers.

MATERIALS AND METHODS

PATIENTS

Tissue samples were separated into two sample groups, here named reference group and study group (see also **Table 1**). The reference group included 36 breast cancer samples from patients diagnosed 1999–2009 with high-grade invasive ductal carcinoma (IDC). Among them, 21 cases were HER2-positive and 15 cases were HER2-negative. The study group included samples from 16 HER2-positive ethnic Danish breast cancers patients. These patients were diagnosed from 2005 to 2008 and had their primary tumor surgically removed and received adjuvant trastuzumab (trastuzumab/Herceptin was obtained from Roche). After surgery, the patients received adjuvant chemotherapy, irradiation, and antihormonal treatment according to national standards, and eventually trastuzumab. All patients received trastuzumab every third week for one year. None of the patients had been treated for breast cancer previously. Eight patients developed recurrent disease with distant metastases after 5–8 years of follow up. In the present study, we considered these patients as resistant to trastuzumab. Patients without recurrence were considered sensitive. All samples were obtained from the local tissue bank (Herlev Hospital, Copenhagen, Denmark) as formalin-fixed paraffin-embedded (FFPE, here fixed within 1 h of surgery by 24–72 h incubation in formalin at room temperature). Tumor grade was based on HE stained sections. The study was performed in accordance with permission given by The Regional Scientific-Ethical Committee for Copenhagen, Denmark (J. nr. H-3-2014-010).

Table 1 | Patients included in this study.

	n	Age (range)	Diagnosis year	HER2 neg (*n*)	HER2 pos (*n*)
Reference group	36	62 (36–92)	1999–2009	15	21
Study group	16	51 (33–68)	2005–2008	0	16

Age range and year range of diagnosis are indicated for the reference group and study group.

IMMUNOHISTOCHEMISTRY

Immunoperoxidase staining for clinical biomarkers included staining for ER (mAb clone SP1, Dako, Glostrup, Denmark), PR (mAb clone PqR636, Dako), P53 (mAb clone DO7, Dako), Ki67 (mAb clone Mib1, Dako), and HER2 (mAb clone 4B5, Roche). For ER (RTU), PR (mAb diluted 1:100), P53 (mAb diluted 1:200), and Ki67 (mAb diluted 1:200), 3–5 μm tissue sections were pretreated using DakoLink and stained in a Dako immunostainer, whereas HER2 staining was performed using a Ventana instrument (Ventana) according to the manufacturers guidelines. Scores of ER, PR, p53, and Ki67 were the percentage of positive cancer cells. The IHC scores of HER2 were performed in accordance with the HercepTest™ Interpretation Manual – Breast (Dako, Glostrup, Denmark).

HER2 FISH

Cases that scored 2 in HER2 immunoperoxidase staining were additionally tested by FISH analysis using the HER2 FISH pharmDx™ Kit-K331 (Dako, Glostrup, Denmark) according to the manufacturers' instructions.

MicroRNA-21 *IN SITU* HYBRIDIZATION AND SCORING

Automated miR-21 ISH was performed on a Tecan Genepaint instrument (Tecan, Switzerland) essentially as described previously (10). In brief, deparaffinized sections were treated with proteinase-K (25 μg/mL for 8 min at 37°C). Double-FAM-labeled miR-21 and scrambled LNA™ probes (Exiqon, Vedbæk, Denmark) were incubated at 30 nM for 1 h in Exiqon hybridization buffer (Exiqon) at 57°C. The probes were detected with alkaline-phosphatase conjugated anti-FAM (1:800, Roche) for 30 min at 30°C and then incubated with freshly prepared NBT-BCIP substrate containing 0.2 nM levamisole for 1 h at 30°C. For scoring of the miR-21 ISH staining, we obtained digital whole slides using a Hamamatsu scanner (20× objective). The miR-21 staining was scored at the level of cellular tissue compartments, stromal vs. cancer cells, and at the level of staining intensity/density (0, 1, 2, or 3). Score 0, indicated no staining or similar staining as background level, score 1 indicated staining in a subset of cells that could be weak or intense (up to 10%), score 2 indicated staining in a larger subset of cells (10–50% of the cells) that could be weak or intense, and score 3 indicated intense staining in virtually all cells of the tissue compartment (examples of miR-21 scores are shown in **Figure 1**). Scoring was performed independently blinded by two observers (Eva Balslev and Boye Schnack Nielsen). If the difference between scores was more than one, a consensus score was determined. The averages of the two scores are presented.

MicroRNA-21/HER2 DOUBLE STAINING

MicroRNA-21 ISH combined with immunohistochemical staining for HER2 was performed essentially as described (35). After proteinase-K treatment, sections were hybridized with 20 nM

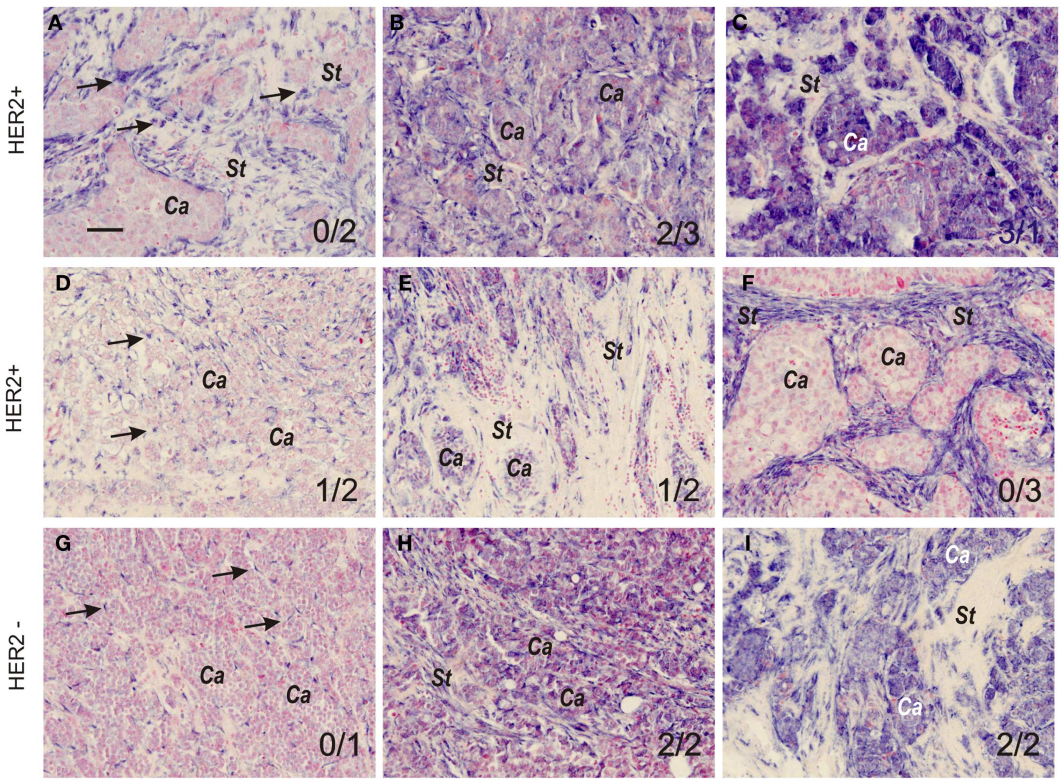

FIGURE 1 | MicroRNA-21 expression patterns and scoring in grade 3 breast cancers. Representative miR-21 ISH expression patterns and intensities in HER2-positive **(A–F)** and HER2-negative tumors **(G–I)**. The miR-21 ISH signal (dark blue staining, examples indicated by arrows in **(A)**) is seen exclusively in stromal cells **(A,D,F,** and **G)**, or in both stromal cells and cancer cells **(B,C,E,H,** and **I)**. Stromal and cancer cell tissue compartments are indicated by *St* and *Ca*, respectively. In **(D,G,** and **H)**, arrows indicate miR-21 positive stromal cells. The variations in miR-21 ISH signal intensity and prevalence of positive cells were scored 0, 1, 2, or 3 by two observers (see Material and Methods section) in both cancer and stromal cells. The miR-21^{CC} and miR-21St scores are indicated (miR-21^{CC}/miR-21St). All sections were counter stained with nuclear fast red. The examples are from tumors in the reference group and are representative for the individual tumors. Bar: 50 μm.

miR-21 LNA probe for 1 h, and the probe was detected with peroxidase-conjugated anti-FAM (Roche) followed by incubation tyramine-signal-amplification (TSA)-Cy5 substrate for 5 min at room temperature. Polyclonal rabbit anti-ErbB-2 (ab2428, AbCam, Cambridge, UK) was incubated at room temperature and detected with Cy3-conjugated goat anti rabbit (Jackson ImmunoResearch, West Grove, PA, USA). Slides were mounted with Antifade Gold with DAPI (Invitrogen).

STATISTICS

Spearman's rank correlation analyses of miR-21 scores and known clinical parameters were conducted to obtain Spearman rank correlation coefficients, *r*. The differences in miR-21 scores between HER2-positive and HER2-negative, and trastuzumab-sensitive and -resistant patient groups were evaluated using the non-parametric Mann–Whitney *U* test. Statistical significance was considered at the 5% probability level ($p < 0.05$). All statistical analyses and calculations were performed with GraphPad Prism 5 (GraphPad Sofware, La Jolla, CA, USA).

RESULTS

PATIENTS

The patient material is briefly described in **Table 1**. The patient samples in the reference group were used to delineate miR-21 expression patterns and intensities in high-grade breast cancers and to establish a miR-21 scoring system. The miR-21 scores were compared with known clinical parameters, including the HER2 status. The samples in the study group were used to evaluate the miR-21 expression patterns and intensities, to obtain miR-21 scores for correlation with recurrence of disease after adjuvant trastuzumab.

MicroRNA-21 EXPRESSION PATTERNS AND SCORING

MicroRNA-21 ISH was performed in parallel on the 36 samples on an automated platform. miR-21 ISH signal was seen in all cases with variation both in staining intensity and in localization. Among the 36 tumors, all showed staining in tumor stromal cells, mostly fibroblasts, but probably also inflammatory and endothelial cells were positive. The stromal miR-21 staining varied from confined focal staining, with most intense staining in the fibroblastic cells surrounding islands of cancer cells, to staining involving most of or the whole tumor stroma. Using a semiquantitative approach, we scored the miR-21 stroma staining (miR-21st) gradually 1, 2, or 3 as described in Material and Methods section (examples in **Figure 1**). miR-21 ISH signal was also prevalent in cancer cells (miR-21^{CC}) in 23 of the cases, and the positive reaction was scored 0, 1, 2, or 3 in terms of staining intensity

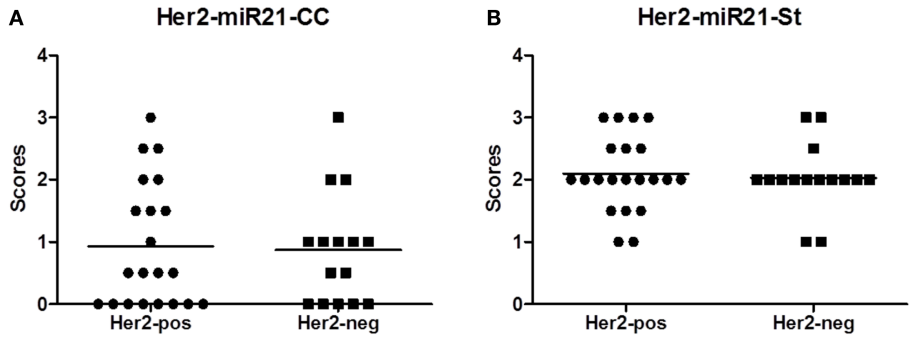

FIGURE 2 | MicroRNA (miR)-21 scores in the HER2 reference group. The miR-21 scoring was performed as described in Material and Methods section, here presented as average scores of two observers: miR-21^{CC} **(A)** and miR-21St **(B)**.

Table 2 | Correlation analyses.

		n	miR-21St	ER	PR	Ki67	P53
miR-21^{CC}	HER2 pos	21	−0.31	−0.07	0.03	−0.24	0.03
	HER2 neg	15	−0.14	0.38	0.56*	−0.04	0.35
	All	36	−0.22	0.07	0.22	−0.15	0.15
		n	**miR-21^{CC}**	**ER**	**PR**	**Ki67**	**P53**
miR-21St	HER2 pos	21	−0.31	0.11	0.19	−0.19	−0.24
	HER2 neg	15	−0.14	−0.26	0.13	−0.17	−0.37
	All	36	−0.22	−0.07	0.14	−0.22	−0.26

*Spearman correlation analyses of miR-21 scores (cancer cell expression, miR-21^{CC} and stromal expression, miR-21St) and available clinical parameters here separated into HER2-negative and positive tumors (Spearman correlations, *$p < 0.05$).*

(examples in **Figure 1**). In general, miR-21 ISH signal was equally intense in all stained tumor cells in the same tumor, however, some variations were observed. Most dramatic variation was seen in two cases (both HER2-negative) in which one tumor cell compartment showed strong staining, whereas an adjacent tumor cell compartment showed virtually no staining (data not shown). In these two cases the scores presented are those with highest staining intensity. Intensely stained myoepithelial cells surrounding foci of carcinoma *in situ* (CIS) were seen in 8 of the 36 cases (data not shown). No ISH signal was obtained with the scramble probe in any of the samples. Additional specificity analyses of the miR-21 ISH probe in breast cancer samples have been performed previously (12).

MicroRNA-21 LOCALIZATION AND HER2 STATUS AND OTHER CLINICAL PARAMETERS

There was no obvious difference in the miR-21^{CC} expression pattern or miR-21St expression pattern when comparing HER2-positive with HER2-negative cases (**Figure 2**), suggesting that the miR-21 expression pattern is independent of the HER2 status. A series of additional molecular parameters were obtained from the cohort by immunohistochemistry: ER, PR, Ki67, and p53 and then correlated with the miR-21 scores (**Table 2**). In HER2-negative tumors, we noted a significant positive correlation ($p = 0.04$) between miR-21^{CC} and elevated PR. Otherwise, none of these parameters showed significant correlation with the two miR-21 scores, miR-21^{CC} and miR-21St. We have previously reported that increased stromal miR-21 levels in grade 1 and 2 lesions are associated with increased cancer cell proliferation as measured by the Ki67 index (12). We did not see a similar correlation in these grade 3 lesions.

MicroRNA-21 CO-LOCALIZES WITH HER2

In order to perform double fluorescence analysis of HER2 and miR-21, we employed a polyclonal antibody, which was compatible with the proteinase-K-directed antigen retrieval needed for miR ISH on FFPE samples. Six samples from the reference group were selected for miR-21/HER2 double immunofluorescence based on (1) the intense staining for HER2 with the polyclonal antibody and (2) the miR-21^{CC} and/or miR-21St positive staining. As expected, we found miR-21 ISH signal in HER2-positive cancer cells (**Figure 3**). We noted that the miR-21 staining intensity in HER2-positive cancer cells varied from absent to strongly positive. For comparison, in a case with prevalent stromal miR-21, we found miR-21 positive stromal cells surrounding HER2-positive clusters of cancers cells (**Figure 3**).

MicroRNA-21 STAINING PATTERNS AND RECURRENCE AFTER HER2-DIRECTED THERAPY

MicroRNA-21 ISH was then performed in parallel on the 16 HER2-positive cases with known disease status after adjuvant trastuzumab. Eight of the patients experienced no recurrent disease after treatment within the 5–8 years follow-up period (sensitive tumors), whereas the other eight patients experienced recurrent disease (resistant tumors). Examination of the miR-21 staining patterns in these samples indicated expression patterns similar to those observed in the *reference group*, thus both tumors with predominant miR-21^{CC} and miR-21St were present (**Figure 4**). However, there was no significant difference in the miR-21^{CC} and miR-21St as well as in the summarized total average miR-21 scores, when comparing resistant and sensitive tumors (Mann–Whitney *U* test). Thus, we found no indications that miR-21 expression patterns could help to identify HER2-positive patients resistant to adjuvant trastuzumab in this study group.

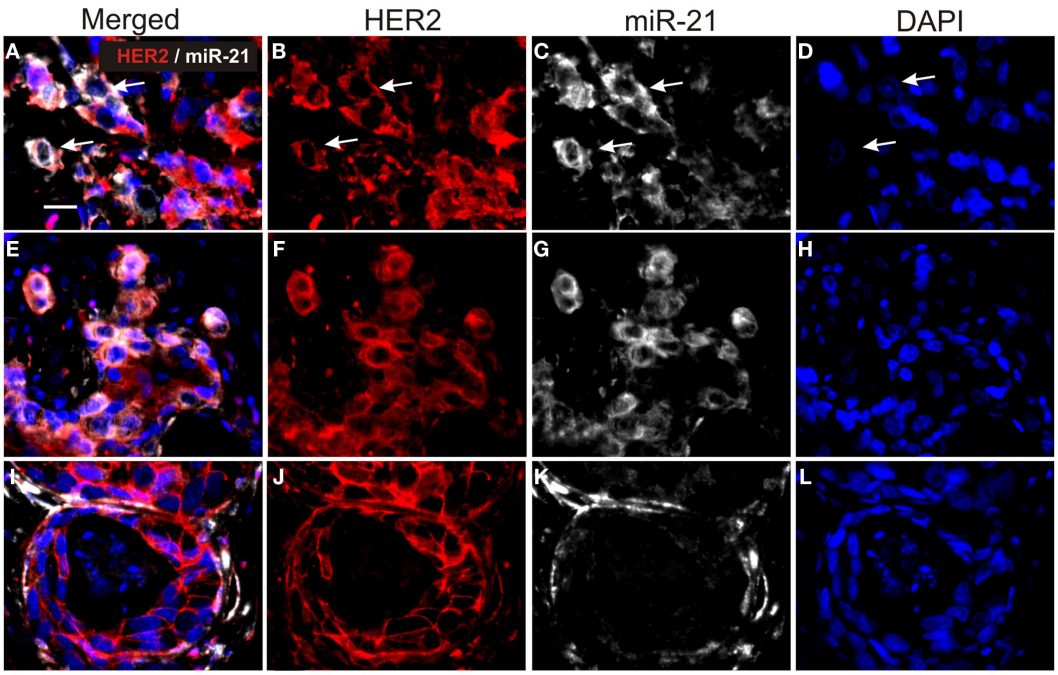

FIGURE 3 | Double staining for miR-21 and HER2 in HER2-positive breast cancers. Tissue sections were processed first for miR-21 ISH and subsequently for HER2 IHC. The figure shows three cases **(A–D)**, **(E–H)** and **(I–L)**. miR-21 was detected using TSA-Cy5 substrate (white) and HER2 with Cy3-conjugated anti-rabbit antibody (red). All sections were counterstained with DAPI (blue). miR-21 ISH signal is seen in HER2-positive cancer cells in cases **(A–D)** and **(E–H)** [examples are indicated by arrows in **(A–D)**], whereas HER2-positive cancer cells in case **(I–L)** are miR-21-negative. The examples are from tumors in the reference group and are representative for the three tumors. Bars: 20 μm.

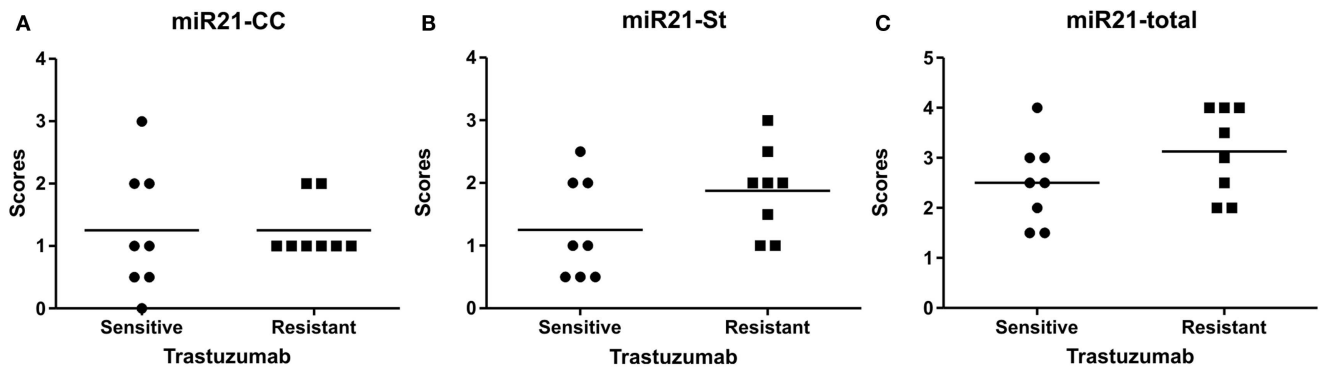

FIGURE 4 | MicroRNA-21 scores in the HER2 study group. The miR-21 scores for cancer cell-associated miR-21 expression, miR-21^{CC} **(A)**, miR-21 stromal expression, miR-21St **(B)**, and the total summarized miR-21 scores **(C)**. The scores are averages of two observers and here presented in scatter grams. Mann–Whitney *U* test: $p = 0.69$ for miR-21^{CC}, $p = 0.13$ for miR-21St, and $p = 0.20$ for total miR-21.

DISCUSSION

MicroRNA-21 is one of the most prevalent miRs in solid tumors, and increased expression levels are associated with poor prognosis both in untreated early-stage colon cancer patients (8, 10, 15) and in breast and colon cancer patients treated with chemotherapy (8, 9, 36, 37). In addition, several recent studies have suggested that miR-21 confers drug resistance to cancer cells *in vitro* (38, 39), including resistance to trastuzumab treatment of HER2-positive patients (19). We assumed that if miR-21 indeed confers drug resistance in HER2 breast cancer patients, knowing the specific cellular origin of miR-21 in the tumors may help to predict response to HER2-directed therapy. In this study, we show that miR-21 is highly expressed in high-grade breast cancers, both in HER2-positive and negative cases, and that miR-21 ISH signal can be seen in both stromal cells and cancer cells in varying expression levels.

Gong et al. (19) found increased miR-21 ISH signal in resistant tumors before and after trastuzumab-directed therapy, and showed that miR-21 antisense oligonucleotides could restore

trastuzumab sensitivity in resistant cells by inducing PTEN expression, suggesting that miR-21 mediates trastuzumab resistance and that antagonizing miR-21 in a therapeutic setting could sensitize cancer cells to HER2-directed therapy. Indeed, we found that both miR-21 ISH signal and HER2 immunoreactivity are seen in the same cancer cells, which would be a prerequisite for successful combination of anti-miR-21 and HER2-directed therapy. In order to assess if miR-21^{CC} expression could predict the response to trastuzumab therapy, we examined the cellular expression of miR-21^{CC} in 16 HER2-positive tumors, half of which relapsed within the 5–8 years follow-up period after adjuvant trastuzumab. However, since we also found miR-21^{CC} expression in trastuzumab-sensitive tumors at the same frequency, our findings do not indicate that high miR-21^{CC} can predict recurrence. Based on our studies of 16 patients treated in the adjuvant setting, our observations imply that miR-21 did not confer trastuzumab resistance, a finding which appears inconsistent with the conclusion drawn by Gong et al. (19), who investigated 32 patients treated in the neoadjuvant setting. However, the major difference in treatment protocols of the two studies does not justify a direct comparison of the results.

Although the relatively small sample size offered little statistical strength to our observations, it should be noted that the concomitant adjuvant chemotherapy may have been a confounding factor that prevented identification of a direct link between miR-21 expression and trastuzumab resistance. Likewise, small patient groups will be relatively sensitive to genetic variation, which in our case may also have contributed to cover the impact of miR-21 as a predictive biomarker. Another parameter that may have prevented identification of a direct link between miR-21 and trastuzumab resistance is the semiquantitative scoring approach employed. In order to minimize experimental variation in staining intensities, all sections, both in the reference group and in the study group, were processed identically and stained in parallel on an automated platform. Manual scoring was then performed on digital whole slides by two observers, and since considerable variation between samples could easily be discriminated, we assumed that our scoring approach was not a limiting factor. Furthermore, a manual scoring approach could potentially be implemented in the daily diagnosis. In previous studies, we used image analysis to obtain quantitative estimates of miR-21 expression (10, 12, 15), but common for those studies, the expression patterns were more homogenous. Here, the cellular origin of expression was a key parameter. It cannot be excluded that alternative semiquantitative approaches may have classified the cases better. For example, RT-qPCR analyses of microdissected tissue compartments, stroma and cancer cells, may potentially provide useful quantitative estimates of the miR-21 expression. Thus, more studies, including examination of the primary and recurrent tumors in both the adjuvant and neoadjuvant settings, are needed to better address how the miR-21^{CC} (and miR-21St) parameters potentially can be used as predictive biomarkers.

In our previous study of miR-21 in grade 1–2 breast cancers (12), none of the 24 included cases showed noteworthy expression in the cancer cells. In the current study, 9 of the 36 cases (25%) showed intense miR-21^{CC} (miR-21^{CC} score 2 or above). These miR-21^{CC} cases included both hormone receptor-positive and -negative cases. Furthermore, miR-21^{CC} did not correlate with any of the other clinical parameters analyzed in the HER2-positive tumors. In HER2-negative tumors, we found a significant positive correlation between miR-21^{CC} and PR. The significance of this association will need to be further explored. Because miR-21 was found strongly expressed in myoepithelial cells surrounding occasional CIS structures in some of the lesions, it is tempting to speculate whether the miR-21^{CC} cases were all of the basal subtype. However, this is unlikely since some of the miR-21^{CC} cases were ER-positive. Thus, our data suggest that the miR-21^{CC} lesions represent an independent group of grade 3 breast cancers. The apparent variation in the cellular expression pattern of miR-21 is an important feature that may impact on targeted therapy since miR-21 in different cell populations may have different (mRNA) targets. Sempere et al. (40) analyzed breast cancers of various grades and hormone receptor status and reported frequent expression in cancer cells as well as expression in the breast cancer-associated fibroblast in some cases. More studies are needed to better establish whether high miR-21 expression in cancer cells is confined to grade 3 breast cancers.

As mentioned above, increased miR-21 expression levels are associated with adverse prognosis in several types of cancer. Experimental studies of tumor models in mice support these findings. In a K-ras-dependent lung cancer model, overexpression of miR-21 caused increased tumor formation, whereas miR-21 deficiency reduced the tumor formation (41). In a skin carcinogenesis model, miR-21 deficiency resulted in reduced papilloma formation (42). In addition, mice lacking the tumor suppressor p53 showed reduced tumor incidence if also lacking miR-21 (43). Thus, based on both clinical and experimental findings, miR-21 is likely a positive driver in the oncogenic process.

Two of the best described miR-21 targets are the tumor suppressors PDCD4 and PTEN. In the context of this study, it is notable that Huang et al. (44) found that activation of HER2 up-regulates miR-21 in breast cancer cell lines and causes successive down-regulation of PDCD4 preventing the tumor cells to enter apoptosis. However, PDCD4 is likely a multifunctional protein, whose role in cancer is only partly understood. PDCD4 was originally found as a nuclear antigen of proliferating cells (45), while others found PDCD4 associated with apoptosis (46, 47). In addition, PDCD4 was reported to be an inhibitor of neoplastic transformation and metastasis (48, 49). In breast cancer tissue, PDCD4 is seen in both normal and malignant epithelial cells and localizes to the nuclei and/or the cytoplasm (50). It remains to be established if miR-21 is involved in the regulation of all of these pivotal functions of PDCD4. Like PDCD4, PTEN has been found in relation to cell proliferation and apoptosis (51), and its presence has been linked to drug resistance (30). Lack of PTEN in breast tumor stroma has, in model systems, been found to strongly enhance transformation of the mammary epithelium (52, 53). In our study, high levels of miR-21 in the breast cancer stroma, which would cause miR-21-directed loss of stromal PTEN, were weakly ($p = 0.13$) associated with resistance to adjuvant trastuzumab. Trastuzumab resistance was reported in patients with breast cancers that were PTEN-negative in immunohistochemistry (30), however, a systematic delineation of the presence of PTEN in different cellular compartments in breast cancer lesions is highly warranted, particularly in

the light of recent studies implying that drug resistance can be generated through tumor stroma (31–34).

In our previous study of grade 1 and 2 breast cancers (12), we found that high stromal miR-21 levels determined by image analysis correlated significantly with increased Ki67 proliferation index. Quantitative assessment by image analysis of the miR-21 ISH signal in the current grade 3 samples was not accomplished due to the complex expression patterns as discussed above. However, our analysis of the miR-21 expression scores in the stroma or cancer cells in the current grade 3 cancers did not reveal correlation with the Ki67 index.

In conclusion, we have shown that the miR-21 expression patterns in HER2-positive breast cancers are highly variable being present in cancer and/or stromal cells, and not linked to known clinical parameters. In the relatively small group of HER2-positive tumors studied, our miR-21 ISH analyses did not contribute significantly to the identification of patients with recurrent disease after adjuvant trastuzumab. We noted that both miR-21^{CC} and miR-21St were independent of ER, PR, Ki67, and p53, suggesting that more extensive studies on trastuzumab resistance are warranted across additional subgroups of breast cancer. Molecular markers of trastuzumab resistance like the serum-derived inflammatory biomarkers (34), or expression of PTEN (30), or truncated HER2 (54) have so far not successfully showed to be clinically valuable predictive biomarkers.

ACKNOWLEDGMENTS

The Danish CancerBiobank (DCB) is acknowledged for biological material and for the data regarding handling and storage.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 April 2014; accepted: 19 July 2014; published online: 15 August 2014.
*Citation: Nielsen BS, Balslev E, Poulsen TS, Nielsen D, Møller T, Mortensen CE, Holmstrøm K and Høgdall E (2014) miR-21 expression in cancer cells may not predict resistance to adjuvant trastuzumab in primary breast cancer. Front. Oncol. **4**:207. doi: 10.3389/fonc.2014.00207*
This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Oncology.

frontiers in
PHARMACOLOGY

CLINICAL CASE STUDY ARTICLE
published: 16 April 2014
doi: 10.3389/fphar.2014.00076

A practical approach to aid physician interpretation of clinically actionable predictive biomarker results in a multi-platform tumor profiling service

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Patients in whom the standard of care has failed or who have uncommon tumors for which no standard of care exists are often treated with drugs selected based on the physician's best guess. The rate of success for this method is generally low. With the advent of fast, affordable tumor profiling technologies, and a growth in the understanding of predictive biomarkers, it is now possible to identify drugs potentially associated with clinical benefit for such patients. We present the Caris approach to evidence-based tumor profiling and two patients with advanced ovarian and prostate cancer in whom standard of care had failed and tumor profiling identified an effective treatment schedule. To establish Caris Molecular Intelligence™ (CMI), over 120,000 clinical publications were screened and graded to characterize the predictive value of biomarkers that form the panel of tests. CMI includes multiple technologies to measure changes in proteins, ribonucleic acid, and deoxyribonucleic acid and proprietary software that matches the test results with the published evidence. The CMI results enable physicians to select drugs that are more likely to benefit the patients, avoid drugs that are not likely to work, and find treatment options that otherwise would not be considered. Worldwide, over 60,000 cancer patients have undergone evidence-based tumor profiling with CMI. In the cases reported in this article, CMI identified treatments that would not have been routinely used in the respective clinical setting. The clinical outcomes observed help to illustrate the utility of this approach.

Keywords: evidence-guided, personalized medicine, biomarkers, oncology

INTRODUCTION

Patients who reach the end of their guidelines-defined treatment options and are suitable and willing to receive further treatment, or who present with an uncommon cancer type where treatment options are limited, are among the most difficult to treat. In such cases, the treatment decision is based on the individual patient's clinical context, physician's experience and clinical judgment, local practice guidelines and the patient's medical and treatment history. While the overall rate of success for cancer drug treatment has been estimated at 35% (Jackson, 2009), treatment for these patients is less effective, e.g., the response rate to therapy in patients meeting the inclusion criteria for early clinical studies is around 10% (Olmos et al., 2012).

Advances in the discovery of prognostic and predictive biomarkers can provide oncologists with vital information which helps to stratify their patients for risk of tumor progression and identify potentially beneficial therapeutic agents based on biomarker expression patterns. For example, lung cancer has traditionally been viewed as difficult to treat and associated with poor prognosis. The last 5 years has seen epidermal growth factor receptor (EGFR) mutation testing become standard of care for selection of treatment with erlotinib or gefitinib in non-small cell lung cancer (NSCLC). Many companion diagnostics are now part of the approved drug label and clinical guidelines indicate which specific biomarker should be assessed in a consistent manner in all patients prior to treatment selection in certain tumor types in order to identify a defined subgroup for which the respective treatment is indicated.

While many new drugs received regulatory approval together with companion diagnostics in a limited, often lineage specific, clinical setting, the biological principles governing cancer growth can often be extrapolated to other indications as well. One example is the utility of human EGFR 2 (HER2)-directed treatments for patients with lung cancer that have a HER2 (encoded by the *ERBB2* gene) mutation. While this occurs in fewer than 2% of all patients with lung cancer HER2-directed targeted treatments led to disease control in 82% of patients with HER2 mutations (Mazières et al., 2013). This shows that for a patient with no available standard treatment options molecular profiling can reveal specific biomarkers that are associated with benefit from drugs that would typically not have been considered for treatment.

As the majority of individual mutations or other molecular changes are usually rare, a comprehensive profiling increases the chance that a valuable alteration is identified. Comprehensive profiling delivers all relevant information at once, rather than taking a stepwise approach where tests are ordered one by

one dependent on the outcome of single biomarker results. This saves valuable time for the patient and confers an important advantage because the comprehensive overview of the patient's molecular changes provides the best support for rational treatment decisions.

Over recent years, tumor profiling has become a standard in many large university centers. Tsimberidou et al. (2012) published how tumor profiling guided recruitment of patients into clinical trials with targeted drugs at the MD Anderson Cancer Center. Patients enrolled in studies that required a matching genetic aberration had a clinical response rate of 27% whereas only 5% of patients that could not be assigned to a trial based on molecular profiling responded (Tsimberidou et al., 2012). In a similar approach undertaken at the Princess Margaret Cancer Centre in Toronto, six of twenty-one patients (29%) enrolled into ongoing clinical trials with a therapy matched to a genetic aberration had a confirmed partial response (Bedard et al., 2013). Both of these groups employed large, cross-functional teams of experts to interpret the results from molecular testing. The ability to dedicate experienced teams to guide tumor profiling-directed treatment is beyond the capabilities of most community practices. Therefore alternative methods had to be developed to allow patient's access to reliable and actionable tumor profiling results.

CMI: AN EVIDENCE-BASED APPROACH OF TUMOR PROFILING FOR CLINICAL DECISION-MAKING

Caris Life Sciences started to offer evidence-based molecular profiling over 8 years ago. The approach taken has been constantly refined, resulting in a service which is adapted to the latest scientific knowledge. Caris Molecular Intelligence^{TM} (CMI) supports physicians in implementing actionable results from comprehensive tumor profiling in their routine practice. CMI is performed in a high-throughput laboratory that has been customized to accommodate large numbers of specimens for testing on multiple technology platforms. Comprehensive molecular testing is coupled with an evidence-based proprietary algorithm that translates complex biomarker results into a table of drugs that may provide benefit or lack of benefit for that patient. Caris has received accreditation from Clinical Laboratory Improvement Amendments (CLIAs), as well as an extensive list of certifications from the state of New York. Caris also has recently obtained the molecular profiling industry's first-ever accreditation to the International Standards Organization (ISO) 15189: 2012 "Medical laboratories – Requirements for quality and competence," by the American Association for Laboratory Accreditation (A2LA). The CMI service provides each treating physician with the relevant biomarker testing and current expert interpretation needed to make clinical treatment decisions for each patient.

The CMI service uses a variety of established technology platforms to measure a panel of carefully selected biomarkers including immunohistochemistry (IHC), fluorescent *in situ* hybridization (FISH), polymerase chain reaction (PCR), and direct gene sequencing. Taking an approach that is not reliant on a single technology is critical to perform clinically relevant biomarker testing. Proteins, gene expression, mutations, and gene rearrangements can all have utility as predictive biomarkers. For example, of the 93.5% of compounds predicted to be beneficial which were identified, 87.2% of them were driven by IHC and ISH results, 12.6% by IHC, ISH, and NGS results, and 0.2% driven by NGS results alone. Therefore, an assessment of the molecular profile of a tumor with just one technology will miss potential therapeutic options for the patient.

To keep the biomarker panel current, an ongoing review of the medical literature is performed to review the evidence of predictive associations of biomarkers with available therapeutics. The interpretation of the biomarker evidence is under the governance of a cross-functional group comprised of oncologists, molecular geneticists, pathologists, and research scientists. All biomarkers tested in the CMI service are included based on the strength of their supporting evidence according to a version of the United States Preventative Services Task Force (USPSTF) level of evidence methodology adapted from Harris et al. (2001). The content of each scientific paper is appraised for study design, study validity, and applicability of the biomarker in drug selection. Today, over 95% of drug/biomarker associations included in the service are supported by level 1 (randomized, controlled trials or meta-analyzes) or level 2 (non-randomized, controlled trials, single arm or cohort/case-control analytic studies) evidence. The evidence system used by Caris allows the service to evolve in response to new clinical data. As new markers are identified and vetted, they can be added to the panel quickly. An overview of the evidence process for CMI is shown in **Figure 1**.

The CMI report aligns the molecular profile of the patient's tumor to relevant therapeutic agents associated with potential benefit or potential lack of benefit for the purpose of serving as a decision support aid for the treating physician. The report results are provided to the treating physician and supported by the relevant references from the peer-reviewed literature as determined by the evidence review process. The report also enables the treating clinician to review in detail the biomarker testing that has been performed, as well as link directly to the clinical evidence supporting the biomarker-drug association.

CLINICAL EVIDENCE SUPPORTING USE OF CMI

A manuscript has been published with a number of independent accompanying abstracts which have reported how CMI was used in clinical practice. The Bisgrove study (Von Hoff et al., 2010) was the first clinical trial to assess the use of a multi-platform approach to molecular profiling to identify treatment targets in patients with refractory cancers. In this study, patients were profiled with the CMI panel and physicians chose a treatment regimen based on the results. Clinical benefit was defined as a 30% increase in progression-free-survival (PFS) with molecularly guided treatments, compared to the PFS under the most recent prior regimen. The majority of patients had molecularly identifiable targets and 18 of 66 patients (27%) treated on the basis of molecular profiling derived clinical benefit. A recent study in patients with refractory breast cancer showed that tumor profiling resulted in a revision of the original treatment decision for all patients. Tumor profiling based therapy resulted in a clinical benefit in 52% of heavily pretreated patients (Jameson et al., 2013). A review of all patients

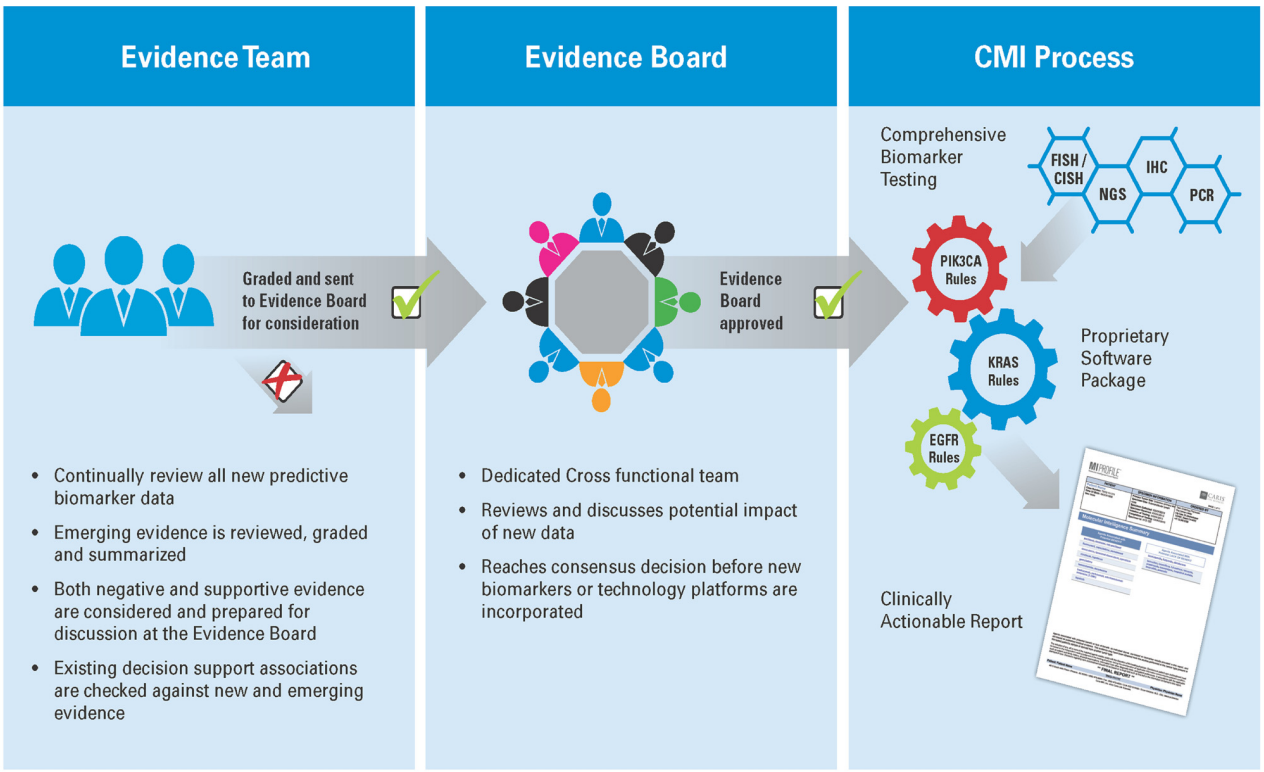

FIGURE 1 | Generating Molecular Intelligence: an extensive literature review is performed by a multi-disciplinary review team, which examines new research and relevant research, and grades it based on US Preventative Task Force methodology in the context of the biomarkers revealed through the profiling services. The output from this evidence review forms the basis of a proprietary software package which is used to translate the results of comprehensive biomarker testing to a clinically actionable report, providing physicians with meaningful biomarkers, actionable drug associations, and relevant clinical trials for their individual patient.

treated in a single center in Australia resulted in clinical and survival benefits in over half of the patients and confirmed the role of molecular profiling in a clinical practice setting (Dean and Wallace, 2013). Though preliminary evidence supports clinical utility, the degree to which CMI improves patient outcomes has not yet been demonstrated conclusively. Further evaluations of the approach are currently ongoing.

CASE STUDY: RESPONSE TO PEMETREXED IN A PATIENT WITH METASTATIC PROSTATE CANCER

A 63-year-old patient was diagnosed with metastatic prostate cancer in 2006 with a Gleason score of 9 and involvement of the pelvic lymph nodes. The patient initially received radiation therapy to the pelvis and the prostate followed by treatment with gonadotropin-releasing hormone (GnRH) analogs. After 4 years the disease progressed and metastasized to the bones, lung, and liver. Treatment with bisphosphonates, sipuleucel-T, abiraterone, enzalutamide, docetaxel, and cabazitaxel did not stop progression. The disease could never be controlled except for short transient partial responses under docetaxel and abiraterone. Embolization of the left liver lobe resulted in temporary local pain relief and as a last resort, the patient received carboplatin with etoposide, which resulted in a transient partial response followed by rapidly progressive disease.

As all guideline-recommended treatment options had failed, it was decided to perform CMI tumor profiling. At this time, the patient had extensive liver metastases with underlying cirrhosis, which caused considerable pain. He was wheelchair bound and unable to walk with an Eastern Cooperative Oncology Group (ECOG) performance status of 3, approaching 4. Within 6 weeks, his prostate specific antigen (PSA) had risen from 84 ng/ml to 177 ng/ml and lactate dehydrogenase (LDH) levels had risen from 569 to 2196 IU/L. All signs and symptoms pointed to a rapidly progressive decline of the patient's general condition.

The CMI report indicated that the tumor was not expressing thymidylate synthase (TS), a protein involved in generation of critical components for DNA synthesis and repair pathways. Published level II evidence from a study of 268 patients with advanced NSCLC who received treatment with pemetrexed after prior chemotherapy found that patients with low TS expression had a longer median PFS compared to those with high TS expression (Chen et al., 2011). The CMI report associated low TS expression with tumor sensitivity to fluoropyrimidines and other folate analogs with potential benefit from 5-fluorouracil, capecitabine or pemetrexed. Therefore, it was decided to begin treatment with single agent pemetrexed at the end of September 2013 based on the physician's choice. The patient's general

condition improved rapidly and the intense right upper quadrant pain resolved completely. Within 5 weeks of starting pemetrexed treatment, a computed tomography (CT) scan showed decrease in size of multiple liver metastases (**Figure 2**). The tumor marker PSA dropped to 5.1 ng/mL and LDH returned to normal (423 IU/L). The patient can now care for himself and his ECOG performance status is 1. The treatment has been tolerated exceptionally well and no further admissions to the hospital became necessary. After 4 months, at this time of this report, the patient continues to receive 3 weekly cycles of pemetrexed, PSA (7.5 ng/mL) and LDH (539 IU/L) are indicating a continued response.

CASE STUDY: RESPONSE TO CETUXIMAB AND IRINOTECAN IN A PATIENT WITH METASTATIC OVARIAN CANCER

A 49-year-old woman was diagnosed with stage IV ovarian cancer in August 2009 after feeling abdominal pain. A CT scan revealed that she had a mass in her right ovary, which was diagnosed as a mixed high grade serous and endometrioid carcinoma. Surgery confirmed metastatic disease and the patient began standard treatment with a combination of intraveneous paclitaxel and carboplatin and intraperitoneal docetaxel/cisplatin. During the time on treatment, the patient had a partial response; her cancer antigen 125 (CA-125) level dropped from 475 to 70 U/mL but did not return to normal levels (less than 35 U/mL). A laparotomy revealed no obvious tumor masses but the persisting elevation of CA-125 indicated residual disease.

As the standard treatments had failed a portion of the initial biopsy material was sent for CMI testing to identify additional treatment options. Based on the findings of the report, the patient was treated with doxorubicin followed by topotecan but both treatments had to be discontinued due to intolerable toxicities. Doxorubicin was selected based on overexpression of topoisomerase 2A (TOP2A), which has been linked with level II evidence to doxorubicin response in breast cancer (Durbecq et al., 2004).

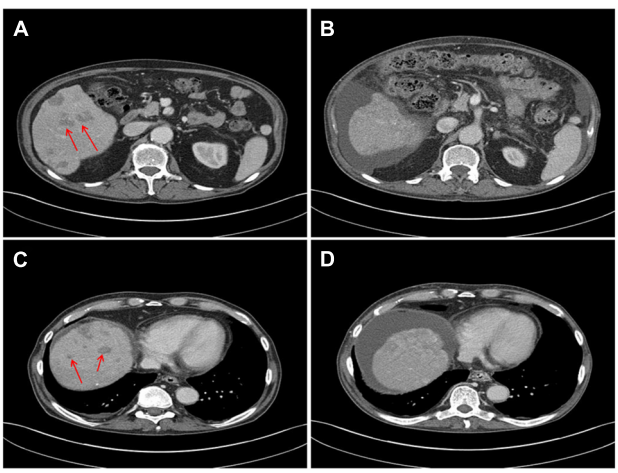

FIGURE 2 | CT scans before (A,C) and 5 weeks after initiation of treatment with pemetrexed (B,D) show decrease in size of metastases and liver sclerosis and ascites due to previous embolization.

In patients with ovarian cancer treated with topotecan, tumors with low or undetectable Topoisomerase 1 (TOPO1) protein levels had a median time to progression of 4.3 months, compared to 13.2 months in patients with high TOPO1 expressing tumors (Litzow et al., 2010). Although poorly tolerated, both treatments resulted in transient decreases in CA-125 to normal levels.

The CMI report also found overexpression of the *EGFR* gene and overexpression of the TOPO1 protein. EGFR gene overexpression along with high phosphatase and tensin homolog (PTEN) protein expression indicated potential efficacy from cetuximab, which is targeting the EGFR receptor (Personeni et al., 2008; Sartore-Bianchi et al., 2009). Irinotecan causes cell killing by blocking TOPO1. In addition to the topotecan evidence cited above, level II evidence in colorectal cancer (CRC) patients treated with irinotecan-containing adjuvant chemotherapy demonstrated that there was a significant improvement in survival in patients who expressed TOPO1 compared to those with low TOPO1 expression (Kostopoulos et al., 2009). These results indicated potential benefit from cetuximab and irinotecan and combination treatment with these drugs was started in late October 2010. After 2 months, bevacizumab was transiently added to this combination (based on overexpression of the hypoxia-inducible factor 1-alpha (*HIF1A*) gene) until it caused toxicity. Within 2 months of the starting the combination of cetuximab and irinotecan the patient's CA-125 level dropped from 64 to 10 and stayed normal over the course of the first 8 months of treatment. Although an attenuated dose was used, toxicities led to discontinuation of therapy. After discontinuation of cetuximab and irinotecan the patient developed progressive disease with pelvic and liver metastases. The patient was then put on treatment with cyclophosphamide, which was not included on the CMI report but resulted in a transient response. Currently the disease is slowly progressive and the patient is still on cyclophosphamide at an attenuated dose.

A significantly long remission after recurrence is unusual in ovarian cancer and irinotecan and cetuximab are rarely used in ovarian cancer. This observation is of significant importance as it justifies further exploration of treatments guided by tumor profiling instead of using histological diagnosis of the tumor alone.

CONCLUSION

Patients with metastatic cancer frequently arrive to a point in their clinical care when all standard of care options have been tried and they require further treatment. Although guidelines recommend that these patients enter either clinical trials or palliative care, they are often fit enough and willing to continue to receive further cytotoxic treatment. Comprehensive tumor profiling can identify active treatment options, help avoiding treatments which are likely not active, and find treatments that otherwise would not have been considered. Meaningful integration of the information generated by comprehensive biomarker testing requires cross-functional expertise to aid interpretation and determine which results are clinically relevant. As this level of support is rarely accessible, CMI provides a service which helps physicians to develop evidence-based treatment plans. CMI combines the results of tumor profiling with a thorough assessment of the

published clinical evidence in a comprehensive report that includes drugs associated with benefit, lack of benefit as well clinical trials that may be relevant for the patient. The clinical experience with tumor profiling in routine clinical practice has been promising.

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Conflict of Interest Statement: Kenneth Russell, Andreas Voss, and Todd Maney are employees of Caris Life Sciences. Drs. Shunyakov and Dicke did not have any commercial or financial relationships that could be construed as potential conflicts of interest.

Received: 06 February 2014; accepted: 30 March 2014; published online: 16 April 2014. Citation: Russell K, Shunyakov L, Dicke KA, Maney T and Voss A (2014) A practical approach to aid physician interpretation of clinically actionable predictive biomarker results in a multi-platform tumor profiling service. Front. Pharmacol. ***5****:76. doi: 10.3389/fphar.2014.00076*

This article was submitted to Pharmacology of Anti-Cancer Drugs, a section of the journal Frontiers in Pharmacology.