Finding biomarkers of resistance to targeted cancer therapies

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Abstract

In the past decade we have witnessed the emergence of a completely new class of cancer drugs: the targeted therapeutics. Such drugs are potentially far more powerful than the conventional broadly-acting chemotherapeutic agents as they target cancer-specific lesions. However, in spite of their often superior efficacy and limited toxicity, some of the old problems associated with the use of the previous generation of cancer drugs persist; most notably development of therapy resistance. Since these new drugs inhibit cancer-relevant signalling pathways in a highly specific fashion, only a limited number of genetic events may enable cancer cells to bypass such a specific block in proliferation. It is therefore likely that the molecular pathways that can cause resistance to such targeted therapies are similar *in vitro* and *in vivo*. Consequently, it should be possible to uncover mechanisms of resistance to targeted therapies in suitable pre-clinical models. In particular, unbiased genetic approaches in drug-sensitive cancer cell lines may be useful to identify candidate biomarkers of therapy resistance. Here I discuss the new genetic tools that have become available to perform such studies with an emphasis on the applications of these technologies to identify biomarkers of resistance to targeted therapies.

Introduction. A new generation of targeted therapeutics for cancer: the need for companion diagnostics

Our increased understanding of the molecular defects that underlie oncogenesis has begun to result in a shift away from the broadly-acting cytotoxic drugs as it has sparked the development of a completely new generation of targeted therapeutics that specifically inhibit the genetic defects that underlie the oncogenic process [1]. Such drugs are generally believed to be very effective because tumours tend to become 'addicted' to activated oncogenic signalling pathways and sudden drug-induced interruption of the signal to which the cells have become addicted leads to rapid cell death [2]. Perhaps the most salient example of this novel approach to cancer therapeutics is the development of a specific small molecule inhibitor of the BCR-ABL kinase that gives rise to chronic myeloid leukaemia (CML). This drug, Imatinib mesylate (Gleevec), has been shown to be exceptionally potent in treating this disease with very few side effects [3]. It is widely believed that in the next 5 to 10 years the clinical application of these new targeted therapeutic agents will personalise, and thereby revolutionise, the care for cancer patients [4]. A more individualised approach to the treatment of cancer with targeted therapies necessitates the development of companion diagnostics to identify the correct patient subgroup eligible for a particular targeted therapy. Not only will the new targeted therapies be far more effective and less toxic than the conventional chemotherapeutic agents, the new generation of molecular diagnostics will likewise have more far-reaching consequences for patient management than conventional diagnostics, which is often limited to 'grading' and 'staging' (Fig. 1). Not surprisingly, regulatory authorities, most notably the US Food and Drug Administration, have taken a keen interest in regulating such new and often complex (multi parameter) diagnostic tests (referred to as In Vitro Diagnostic Multi Index Assays, IVDMIAs). This regulatory oversight is urgently needed, as these new tests will often be used to guide therapy choice. A highly undesirable side effect of almost all targeted therapies is their high cost. To make a bad situation worse, a recent development is the combination of multiple such expensive targeted therapies, which can easily raise the cost of therapy above $100,000 per patient per year. To keep the healthcare system affordable, the development of advanced diagnostic tests to identify likely responders to expensive therapies is urgently needed. However, the development of such diagnostic tests to identify (non)responders has received considerably less attention over the past

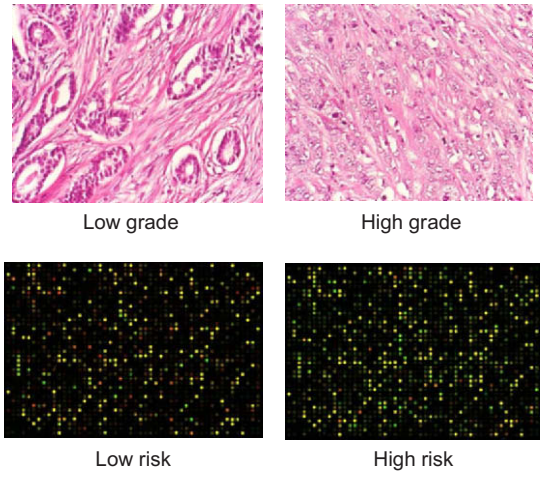

Fig. 1. Old versus new diagnostics for cancer. Conventional diagnostics rely heavily on morphologic criteria to judge cancer aggressiveness ('grading'). More recently, multi gene tests (so called In Vitro Diagnostic Multigene Index Assays, IVDMIAs) (shown is a microarray slide of a MammaPrint® breast cancer prognosis test [5]) have been shown to be powerful tools to predict disease outcome and have become subject to scrutiny by the US Food and Drug Administration.

decade than the development of new targeted therapies. Here I discuss how such predictive diagnostic tests can be developed, through unbiased identification of biomarkers of resistance to therapy.

Biomarkers of resistance to conventional cancer therapy

The responses to conventional chemotherapeutic agents are in general hard to predict by any type of diagnostic test. This is most likely due to the fact that these agents are pleiotropic in their actions and multiple mechanisms can cause resistance to therapy. It is probably why there is a multitude of 'prognostic' gene expression signatures (that foretell recurrences) but far fewer 'predictive' gene signatures that are powerful indicators of therapy response (Fig. 2). One problem in the generation of such predictive signatures is that patient material is often not readily available. Patients that are treated for metastatic cancer usually do not have biopsies taken prior to therapy. Furthermore, patients treated in the metastatic setting often receive combination therapies, which makes it difficult to know for sure to which drug in a cocktail a patient responded. One way around these issues is the use of gene expression profiling in a neo-adjuvant setting – a protocol in which patients are treated with chemotherapy prior to surgery. Short-term responses to drugs can readily be measured in this setting and tissue biopsies from primary tumours can be obtained relatively easily before and after therapy

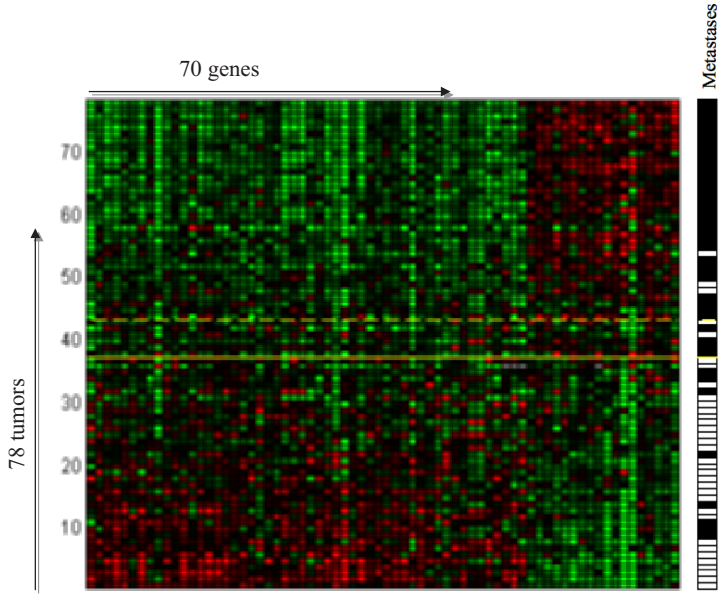

Fig. 2. Predicting disease outcome by complex gene tests. Example of a mutli-gene test that foretells disease progression in primary breast cancer. A set of 70 genes (vertical columns) is used to classify 78 primary breast cancers (horizontal lines) according to their risk of future distant metastases. The bar on the right indicates the actual outcome of disease (white = metastasis, black = no metastasis). Adapted from van 't Veer and colleagues, [6].

(see e.g. [7]). Others have chosen to use *in vitro* models to identify genes involved in drug resistance. For instance, several groups have used gene expression profiling on series of human cancer cell lines of known drug sensitivity to identify patterns of gene expression that correlate with therapy response *in vitro* [8,9]. However, the validation of such *in vitro* generated signatures is still very limited. A more general problem with therapy resistance signatures is that physicians often do not have alternative therapies and are not going to withhold their standard therapy because a biomarker indicates that the therapy 'probably will not be effective'. Thus, the sensitivity and specificity of these predictive signatures will have to improve significantly before they will be of clinical utility.

An interesting recent finding is that in a number of types of cancer, chemotherapy induces a post mitotic state that resembles that of replicative senescence [10]. Such chemotherapy-induced senescent state is characterised by a typical cell morphology, a lack of proliferation and expression of a number of senescence-associated markers [11]. Since replicative senescence was recently shown to be a reversible state of growth arrest [12] (as opposed to irreversible, which was the model until recently), it is also likely that chemotherapy-induced senescence is reversible. This has important consequences for the question whether chemotherapy-induced senescence is a potentially useful biomaker for therapy response. On the one hand one could argue that induction of senescence is favourable, as it halts proliferation. On the other hand one could envision that induction of a reversible state of growth arrest allows the cancer cells to 'duck' the brunt of the chemotherapy by temporary withdrawal from the cell cycle and resume proliferation when the worst is over. It is therefore at this point an open question whether chemotherapy-induced senescence is a marker of good or poor response to therapy. Either way, it is likely that chemotherapy induced senescence will have some predictive value.

Biomarkers of resistance to targeted cancer therapies

Historically, the most effective therapies have been empirically found in large clinical studies. Through trial and error, second line and third line therapies have been identified when resistance against first line therapy develops. The elucidation of the many signalling pathways in cancer provides some rationale for the sequential use of different classes of conventional cancer drugs. When a cancer cell develops resistance to a DNA damaging agent (becomes resistant to the DNA damage checkpoint) the cell might still have an intact mitotic checkpoint and hence might respond to, for instance, a microtubule disrupting agent. Nevertheless, the use of conventional chemotherapy follows mostly the 'one size fits all' scheme: for breast cancer, combination 'A' is used, for lung cancer combination 'B', etc. Such standardised protocols per cancer type ignore one of the most important lessons from two decades of molecular genetics research in cancer, namely that each tumour has a unique set of genetic alterations (between five and ten) that drive the oncogenic proliferation of the cell. In the future, identification of these altered pathways in each individual tumour will be crucial to assign the optimal targeted therapeutic regimen to each individual patient. In the next decade, we will witness a move from describing cancers according to their tissue of origin (e.g. breast cancer, colon cancer) to describing them by the major pathway that drives their proliferation (e.g. EGFR-driven cancer, PI3K-driven cancer), irrespective of their tissue site of origin. Such a nomenclature will greatly facilitate the optimal selection of the pathway-targeted therapy of choice of each individual tumour. Indeed, it is already routine practice for considerable time to classify breast tumours as 'oestrogen receptor positive' (ER+) or 'oestrogen receptor negative' (ER–) and this classification is used to decide on the use of anti hormonal therapy. Since anti hormonal therapy for breast cancer has existed for almost 30 years, neither the concept of targeted therapy, nor the concept of naming tumours by the pathways that drive their proliferation is particularly new in principle. The main reason why it has not been adopted on a larger scale is because in most cancers the 'driver' of the oncogenic process is not clear. However, a recent finding is that activation of specific signalling pathways leads to characteristic changes in gene expression, which can be identified using DNA microarray technology. For instance, the group of Nevins [13,14] has identified such 'pathway signatures' and these might be used to both identify the major signalling pathways that drive proliferation and subsequently also to select specific therapies that target the activated pathway. Such complex gene signatures may also be used to predict responsiveness to therapy. Even though ER status as judged by immunohistochemistry is a powerful predictor of response to hormonal therapy in breast cancer, not all ER-positive patients respond equally well. Obviously, the mere presence of an immunoreactive epitope of ER does not guarantee that the receptor is functional. Therefore, attempts have been undertaken to create gene expression signatures

that predict responses to hormonal therapy in breast cancer. Indeed, the first such profiles have been discovered and may provide additional benefit in the choice of therapy for breast cancer [15].

Another example of a successful targeted therapeutic agent is the humanised monoclonal antibody trastuzumab (Herceptin®) targeting the HER2 growth factor receptor [16]. HER2 is over-expressed in some 20–30% of all primary breast cancers and its expression is correlated with aggressive disease and poor survival [17]. Trastuzumab has shown efficacy in breast cancers over-expressing ErbB2. Patients having amplified HER2 (FISH-positive) breast cancer and those that have tumours that stain intensely in immunohistochemistry (3+) respond better than those that are FISH-negative or stain less intensely for HER2 (2+) [18]. This suggests that those tumours in which HER2 is amplified depend more on the proliferative stimulus that emanates from the receptor than those that do not have amplified copies of the gene. However, only less than 35% of patients with HER2-expressing metastatic breast cancer respond to trastuzumab as a single agent and it is still largely unclear why the majority of the breast cancer patients that over-express the HER2 target are non-responsive to trastuzumab therapy. Identification of drug-unresponsive tumours is desirable as some 5% of patients experience severe side-effects from trastuzumab therapy, including cardiac dysfunction, and 40% experience other adverse effects, such as skin rashes [18,19]. In addition, the cost of these expensive drugs rapidly becomes prohibitive for the health care system and a stratification of those patients that likely benefit most from these drugs is also urgently needed from a health-economic perspective. Recently, Nagata and colleagues showed that one of the earliest responses to trastuzumab therapy is activation of the dual-specific phosphatase PTEN [20]. Their data indicate that this effect on PTEN is relevant to the anti-cancer effect of trastuzumab. More importantly, PTEN expression is lost in over 40% of breast cancers [21] and preliminary data from Nagata and colleagues show that PTEN-deficient breast cancers had significantly poorer responses to trastuzumab-based therapy than those with normal levels of PTEN [20]. That PTEN is a predictor of trastuzumab responsiveness has recently been confirmed by others [22] (our unpublished data). Since PTEN is a negative regulator of PI3K catalytic subunit PIK3CA, this work may suggest that combining trastuzumab with specific PI3K inhibitors could overcome resistance to trastuzumab. Furthermore, since activating mutations in PIK3CA are found in some 25% of all primary breast cancers [23], the mutation status of PIK3CA may have additional predictive power to identify non-responders to trastuzumab-based therapies. Since both PTEN loss and activating mutations in PIK3CA have a very similar downstream biochemical effect, it is likely that a characteristic pattern of gene expression can be found that is associated with an 'activated PI3K pathway'. Such a pathway signature may turn out to be a powerful predictor of trastuzumab responsiveness in the clinic.

Tools for biomarker discovery *in vitro*

Past studies on conventional chemotherapeutic agents have often shown a relatively poor correlation between *in vitro* and *in vivo* drug sensitivity. As a result, there is general scepticism as to whether the study of drug resistance *in vitro* (in cell lines) has any relevance to the mechanisms that underlie drug resistance in the clinic. While this may be true for conventional chemotherapeutics, there is reason to believe that not the case for targeted therapeutics, as the molecular pathways that can cause resistance to such targeted therapies are likely to be similar *in vitro* and *in vivo*. Most notable was the study of Azam and colleagues, in which they performed a random mutagenesis of the BCR-ABL gene *in vitro* and tested for mutants that conferred resistance to Gleevec *in vitro*. Remarkably, all of the major mutations that are found in the clinic were also recovered from this *in vitro* screen, showing that mechanisms of resistance in cell culture and in the clinic are very similar indeed [24].

The example cited above is an example of an 'unbiased' genetic screen in which the screen in the case described above identifies the relevant amino acids involved in Gleevec resistance in the clinic. Such unbiased genetic screens can also be performed at the level of the genome rather than at the level of the single gene. In principle, two types of genetic screens are feasible in mammalian cells: gain of function genetic screens, in which genes are over-expressed and cells with an altered phenotype are selected. More recently, through the discovery of RNA interference, it has also become possible to perform large-scale loss of function genetic screens in mammalian cells. In such screens, gene activity is suppressed by RNA interference and the phenotype is studied. For a comprehensive review of tools to perform genetic screens in mammalian cells, I refer to the recent review by Brummelkamp and colleagues [25].

We have recently demonstrated the utility of large-scale RNA interference screens to understand mechanisms of drug resistance by identifying genes that cause resistance to a small molecule inhibitor of the physical interaction between p53 and MDM2, named Nutlin3 [26,27]. About half of all human cancers lack functional p53 due to mutation. However, many tumours retain normal p53, whose activation could in principle be used for the eradication of tumour cells. One potential approach to activate p53 in tumour cells is to disrupt the interaction between MDM2 and p53, for instance with the small molecule Nutlin3. However, in theory, activation of p53 should be at least as deleterious to normal cells as it is to tumour cells, as the former are not attenuated in their ability to undergo apoptosis, and are more obedient to growth-inhibitory signals than cancer cells. Remarkably however, Nutlin-3 was reported to have strong anti-tumour effects *in vivo*, but did not appear to have major toxic effects in normal mice [28]. This suggested that the tumour and the normal tissue (even though both are wild type for p53) respond differentially to Nutlin3 exposure. To identify genes that modulate cellular resistance to nutlin-3, we introduced into MCF-7 cells (having wild type p53) by retroviral infection a collection of 23,742 different pRETRO-SUPER vectors designed to target 7,914 human genes for suppression by RNA interference [29]. The infected cells were then exposed to Nutlin-3 and resistant colonies identified. From this study it became clear that inhibition of the expression of 53BP1 conferred resistance to Nutlin3 [30]. This result suggests that Nutlin-3's cytotoxic effect results from its ability to turn a cancer cell specific property, activated DNA damage signalling, into a weakness that can be exploited therapeutically. More generally, these data provide support for the notion that RNA interference studies can significantly accelerate pharmacogenetics, and can be used to reveal cellular components that mediate drug cytotoxicity, resulting in a more complete understanding of drug action and enabling more rational decisions on drug application.

Likewise, one can use gain of function genetic screens to identify biomarkers of drug resistance. In a recent study, we used complex retroviral cDNA libraries to ask which genes we could confer resistance to Histone Deacetylase Inhibitors (HDACI). In this screen, we found two genes whose over-expression confers resitance to HDACI, making such proteins potential biomarkers for HDACI resistance (Epping, *et al*., submitted for publication).

The functional genetic approach to biomarker discovery described above is still in its infancy, but holds great promise for the future, as such biomakers are causally linked to drug resistance. This is in contrast with many earlier 'association' studies, in which such a causal link was lacking. It is reasonable to assume that biomarkers that are causally involved in drug resistance are more powerful biomakers in clinical practice than those that are linked by association only.

Conclusions

As pointed out above, it is a daunting task to find reliable biomarkers that predict unresponsiveness to new experimental therapeutics. DNA microarray experiments have not yet delivered on the promise in the area of predictive gene expression profiles, mostly because there is a paucity of suitable tumour samples to perform comprehensive studies. Large numbers of tumour samples are required in genome-wide microarray studies (between 80 and 100), as the use of small numbers of samples increases the chance that associations between genes and therapy outcome is merely coincidental. One way to circumvent this problem is to refrain from unbiased genome wide studies, but to focus on 'candidate drug response modifier genes', which can be identified experimentally by using *in vitro* gain of function and loss of function genetic screens described above (Fig. 3). Once such candidate genes have been identified, one can ask if their expression is correlated with clinical resistance to the drug-of-interest in the clinic by using tumour samples of cancer patients treated with the drug in question, whose response to therapy is documented. The validation of small numbers of candidate genes requires fewer tumour samples, thereby bypassing one of the most significant bottle necks in the discovery of robust biomarkers: the availability of suitable tumour samples.

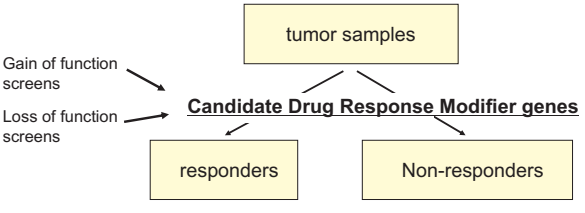

Fig. 3. Schematic outline of functional identification of biomarkers that predict responsiveness to targeted cancer therapeutics. Systematic gain of function and loss of function genetic screens can be performed *in vitro* to identify genes whose up- or downregulation causes resistance to a specific cancer drug. These *in vitro* identified genes are 'candidate drug response modifier genes'. Subsequently, in samples of patients that were treated with the drug (and whose response to the drug is documented) one can verify if these candidate genes have predictive value to identify individual patients that benefit from these drugs.

Conflict of interest statement

None declared.

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