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Biomarkers for therapeutic efficacy


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Abstract

Depending on the tumour type, a larger or smaller number of cancer patients receive chemotherapy with systemic toxicity as the only effect. In that situation, an alternative, not necessarily medical, treatment would have been a better choice – and toxicity (and financial resources) could have been spared by withholding ineffective drugs. One of the reasons for this apparent paradigm is that the tumour cells of each cancer patient may show different sensitivity/resistance towards different chemotherapeutic drugs, i.e. breast cancer or colorectal cancer is not only breast or colorectal cancer. With our increasing biological insight and understanding, it has become apparent that each patient’s tumour tissue is unique and as a consequence, each patient’s tumour cell sensitivity/resistance towards chemotherapeutic drugs may be different. As of today there is no method in routine clinical use to predict the sensitivity/resistance to chemotherapy in its broad sense in the individual patient. This chapter will describe several different DNA, RNA, protein and cell based assay methodologies and marker molecules that have been brought forward as potential predictive assays/markers to be used to select the most effective drugs for the individual cancer patient.

Introduction

Despite the continuous development of new cancer treatment strategies, including optimisation of already known drugs, as well as development of new targeted therapies, too many cancer patients still experience recurrence of their disease with subsequent disease related death. It is thus clear that in order to lower the number of deaths, there is an urgent need for the development of more effective treatment strategies including the introduction of new procedures for optimal prediction of response to treatment. Anti-cancer drug treatment given either alone or in various combinations includes different types of chemotherapy (cytotoxic drugs), endocrine therapy, immunotherapy and recently specified targeted therapies. In most metastatic cancer diseases, the objective response rates (complete and partial responses) to any of these treatment options are far from 100%. This means that a significant number of cancer patients receive chemotherapy with no other effect than systemic toxicity.

While detection of oestrogen and progesterone receptors in breast cancer is a well established routine method to predict objective response to endocrine therapy, there is at present no accepted method to determine whether a cancer patient will benefit from chemotherapy in its broad sense. If such a method existed, it would allow for a tailor-made approach resulting in individualised treatment. This would also imply that for those patients having resistant tumours, such an approach would not only spare them from side effects induced by ineffective chemotherapy, but would also have a major impact on the economics of the health care system in terms of savings of expenses related to otherwise ineffective treatment. Furthermore, it is now clear that predictive molecular assays must be devised before the initiation of clinical trials for new targeted anticancer agents. Use of predictive markers will increase the specificity and usefulness of these
drugs and provide a meaningful clinical evaluation in the population of patients most likely to benefit from the treatment. Thus, the field of cancer drug discovery clearly needs to turn greater attention to the problem of identifying responsive/resistant subsets of patients early in the development process and needs to utilise the knowledge obtained through molecular and cellular studies of cancer biology. The present review will focus on techniques used for identification and determination of predictive biomarkers and will give examples of biomarkers used for selecting cancer treatment to the individual patient. Instead of a lengthy review including all potential markers, this paper will rather give a short introduction to the methodologies that can be used to identify new markers for therapeutic efficacy, followed by selected examples of biomarkers that either already have reached clinical usefulness or are in clinical testing. At the end of the review, we will give our suggestions for clinical validation and implementation of new promising predictive biomarkers in the treatment of cancer patients.

DNA biomarkers

At the cellular level cancer is a genetic disease and the tumour cells have acquired genetic changes that are responsible for the multistep process that drives the malignant transformation [1]. The cancer specific genetic changes may lead to altered mRNA and protein levels and may represent the most important mechanism by which the tumour can permanently acquire new functionality. The acquired specific genetic changes in the cancer cells will, however, not be present in the non-malignant cells of the patient. Therefore, it is obvious to exploit the specific genetic changes of the tumour cells as diagnostic, prognostic and especially predictive tools in the management of cancer patients. The malignant transformation is driven by inactivation of tumour suppressor genes combined with activation of proto-oncogenes. The inactivation of tumour suppressor genes can occur by a variety of mechanisms, including physical deletion, point mutation and/or methylation—all leading to loss of function. Proto-oncogenes, on the other hand, can be activated by amplification, point mutation or structural rearrangements (Fig. 1). Minor changes include point mutations and smaller intragenic deletions and duplications and the DNA amount involved ranges from a single base pair to several millions of base pairs. These changes can be studied by sequencing, LOH (loss of heterozygosity) allelic imbalances (AI) and/or PCR based techniques combined with blotting techniques. The methods are precise for the detection of point mutations, but larger deletions and duplications are not revealed. Only by comparing the DNA sequence of the cancer cells with the normal cells of the patient, is it possible to distinguish point mutations from single nucleotide polymorphisms (SNPs). Polymorphism is a normal genetic variation present in the population and thus not directly linked to the cancer. Further, the gene function may be influenced by epigenetic factors, e.g. methylation.

The major genetic changes include large stretches of DNA, from several thousands to millions of base pairs, and may, depending on methodology, be detected as structural rearrangements or copy number changes (CNC). The techniques described above for detection of minor genetic changes cannot reveal the major genetic changes and vice versa (Fig. 1). The major genetic changes can be studied by CGH (comparative genomic hybridisation), array-CGH and/or FISH (fluorescence in situ hybridization). Using FISH technique, structural rearrangements can be detected as chromosome translocations and the copy number changes are seen as amplifications, deletions, and duplications. FISH methods for detection of these chromosome aberrations were initially developed for cytogenetic specimens (Fig. 2) but have later been refined to cut sections of paraffin embedded tissue (Fig. 3).

The FISH technique comprises hybridising fluorescent labelled probes with target DNA of the cancer tissue. The gene directed probe is preferentially 200–400 kilobase pairs in length and includes the cancer related gene and flanking regions. When selecting the genomic clones for diagnostic use, the gene of interest should be located at a position that excludes or minimises presence of flanking genes that could potentially be related to cancer.

Gene copy number changes can be viewed in the nuclei of a tissue and counted directly. A normal cell will contain two gene copies and a deviation from this number is indicative of an abnormal cell. However, due to the fact that cut sections of tissue are 4–6 μm
Predictive DNA biomarkers can be found by screening cancer cell lines for amplified genes (e.g. HER2), by studying the gene that is the target for the drug (e.g. TOP2A) or by studying genes involved in the pathway of the drug (e.g. EGFR). A number of examples are described below. One of the most well-established biomarkers is the HER2 used for selection of patients for treatment with antibodies directed against HER2. Initially, HER2 (alias of ERBB2) gene amplifications were studied using Southern blotting technique [4], followed by additional information on the mRNA and protein level [5], eventually leading to the development of a treatment targeting the product of the genetic disorder of the tumour [6].

Because the HER2 protein is the therapeutic target, much debate has been devoted to the question about the best use of the different methods, and it is now generally agreed upon [7] that both FISH or IHC (immunohistochemistry) may be used for the assessment of HER2 status in breast cancer. HER2 may, however, be an exceptional case with a very high correlation between the gene copy number studied by FISH and the protein immunoreactivity studied by IHC and precautions should be taken in generalisation from the HER2 case. Very close to the HER2 gene on chromosome 17 is located the TOP2A gene. The gene codes for topoisomerase IIα which is the target for the group of widely used chemotherapeutic drugs, the anthracyclines. The link between the biomarker and the drug was first described in an in vitro grown cancer cell line [8] having amplification of the TOP2A gene and overexpression of the protein, and initially it was reported that the gene copy number and the mRNA and protein level correlated in this lung cancer cell line [9]. Later, studies including samples from

in thickness and the nuclei of a cancer cell is often 10 μm, this simple relationship is not applicable for cut sections. Therefore, a reference probe is added to the probe mix and the ratio between the gene probe (labelled in red) and the reference probe (labelled in green) is scored. Inclusion of a reference probe has the further advantage that gene copy number changes due to polyploidisation of the whole genome can be distinguished from amplifications and allows also the detection of deletions. As reference probe, compensating for the ploidy level of the tumour, the centromere of the chromosome that the gene of interest resides on is often used, although it could be any other region of the genome.

The FISH technique comprises of a few steps (Fig. 4): Pretreatment for making the tissue accessible for the probe, denaturation of probe and target by heating in formamide buffer, overnight hybridisation, stringent wash for removal of unbound probe, and counterstaining and mounting. Specific details and variations of the method are detailed by Nielsen and colleagues [2]. The signals are scored using a fluorescence microscope equipped with filters that are suited for the red and green fluorochromes. A total of 60 nuclei are normally scored, although alternative counting methods can be used [3].

Predictive DNA biomarkers can be found by screening cancer cell lines for amplified genes (e.g. HER2), by studying the gene that is the target for the drug (e.g. TOP2A) or by studying genes involved in the pathway of the drug (e.g. EGFR). A number of examples are described below. One of the most well-established biomarkers is the HER2 used for selection of patients for treatment with antibodies directed against HER2. Initially, HER2 (alias of ERBB2) gene amplifications were studied using Southern blotting technique [4], followed by additional information on the mRNA and protein level [5], eventually leading to the development of a treatment targeting the product of the genetic disorder of the tumour [6]. Because the HER2 protein is the therapeutic target, much debate has been devoted to the question about the best use of the different methods, and it is now generally agreed upon [7] that both FISH or IHC (immunohistochemistry) may be used for the assessment of HER2 status in breast cancer. HER2 may, however, be an exceptional case with a very high correlation between the gene copy number studied by FISH and the protein immunoreactivity studied by IHC and precautions should be taken in generalisation from the HER2 case. Very close to the HER2 gene on chromosome 17 is located the TOP2A gene. The gene codes for topoisomerase IIα which is the target for the group of widely used chemotherapeutic drugs, the anthracyclines. The link between the biomarker and the drug was first described in an in vitro grown cancer cell line [8] having amplification of the TOP2A gene and overexpression of the protein, and initially it was reported that the gene copy number and the mRNA and protein level correlated in this lung cancer cell line [9]. Later, studies including samples from
breast cancer patients have shown that TOP2A gene aberrations are predictive for the outcome of treatment with anthracyclines [10,11] (Fig. 5); however, a direct correlation between gene copy number and protein amount has not been established [12,13]. In contrast to HER2, the DNA and protein measurements are thus not interchangeable in the case of TOP2A and anthracyclines. This relationship is further complicated by the existence of TOP2A deletions.

The epidermal growth factor receptor (EGFR) is amplified or overexpressed in many cancers, including non-small-cell lung cancer and colorectal cancer, and is targeted by EGFR inhibitors. Much effort has been devoted to finding a biomarker that can be used to predict the outcome of treatment with EGFR inhibitors. Initially, IHC, assessing the EGFR protein, was used as a diagnostic tool, and then EGFR mutations were reported, and eventually gene copy number changes were described. Among the various tests developed to predict objective response to EGFR inhibitors, the EGFR FISH test seems to be the most promising [14]; however, only very few studies have included and compared all three methods [15] and a consensus has not yet been reached regarding the use of biomarkers for selection of therapy with EGFR inhibitors. The consensus is also hampered by the fact that different drugs and different cancer types are being studied using different biomarkers (protein, point mutation or major genetic change), different methodologies for the biomarker and even different cut-offs in defining the discrimination between normal (negative) and abnormal (positive) cases.

The lesson learned from the EGFR puzzle must be that future studies of biomarkers for targeted drugs should be well designed and include pilot studies to determine the methodology and cut-off levels to be used in the confirmatory clinical studies. In addition, if the pilot studies cannot point to the right biomarker, both protein and DNA measurements should be included in the relevant clinical trials. Also, it is important to know that point mutations and amplifications, although both being alterations

<table>
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<tr>
<th>Group</th>
<th>n</th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
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<tbody>
<tr>
<td>Deleted</td>
<td>90</td>
<td>0.608</td>
<td>(0.347 - 1.067)</td>
<td>0.0828</td>
</tr>
<tr>
<td>Normal</td>
<td>589</td>
<td>0.936</td>
<td>(0.731 - 1.196)</td>
<td>0.5999</td>
</tr>
<tr>
<td>Amplified</td>
<td>92</td>
<td>0.389</td>
<td>(0.216 - 0.700)</td>
<td>0.0017</td>
</tr>
<tr>
<td>HER2 subgroups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>523</td>
<td>0.631</td>
<td>(0.637 - 1.065)</td>
<td>0.1733</td>
</tr>
<tr>
<td>Positive</td>
<td>244</td>
<td>0.712</td>
<td>(0.501 - 1.011)</td>
<td>0.0576</td>
</tr>
</tbody>
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Fig. 5. Relative effect of Cyclophosphamide, Epirobrin and 5-FU (CEF) in each TOP2A and HER2 subgroup on recurrence free survival (RFS) (Forrest plot). For the primary study endpoint (RFS) in the analyses using the Cox proportional hazard model showed a significant predictive value of TOP2A gene aberrations ($P = 0.016$). The group of patients with TOP2A amplifications had a relative risk reduction of more than 60% when treated with CEF compared to CMF (M = Methotrexate) ($HR = 0.389, P = 0.0017$). Also for the TOP2A deletions a risk reduction was found, however not statistically significant ($HR = 0.608, P = 0.0828$).
of the DNA, may not provide the same biological information. Eventually, it may be a combination of two or more techniques that should be used to determine the biomarker status.

RNA biomarkers

Genetic mutations, genomic losses, amplifications and epigenetic changes including those that control success or failure of chemotherapy, are the driving forces in cancer. The first and currently most easily accessible reporters of these genetic and epigenetic alterations are the downstream transcripts that are under their control. At present, the most commonly used methodology to study expression of multiple genes in cancer tissue is gene expression microarrays. At the turn of the century the development and use of these gene expression microarrays was greatly accelerated with the completion of the code for the human genome, the development of nanotechnology (i.e. miniature robotics and high precision scanners) and the generation of various data analysis tools to allow processing of the multidimensional data generated. Current gene expression arrays contain on a single array multiple detection probes to all annotated mRNA species of the human genome (>25 K mRNA species). Successful gene expression analysis on microarrays and subsequent identification of predictors of disease states involves various steps. First, the tumour sample has to be prepared for analysis on the microarray (Fig. 6; top panel). Therefore, various consecutive tissue sections have to be prepared. Using adjacent HE stained sections, tissue predominantly containing tumour material is selected and from these selected sections ribonucleic acid molecules are extracted. In general, fresh-frozen tumour material is used for gene expression analysis since high quality RNA is needed for this to be successful. However, today, it is also possible to extract RNA from formalin-fixed paraffin embedded material. It should be mentioned, however, that this RNA is fragmented and partially chemically modified and therefore currently less useful for microarray discovery studies. This material is, however, highly suited for validations of gene signatures using e.g. quantitative RT-PCR analysis of selected genes. After RNA extraction, the RNA is quantified and checked for integrity by monitoring the presence of the ribosomal 18S and 28S RNA species and the absence of RNA degradation products. After that, RNA of sufficient quality is reverse transcribed to DNA (called copy DNA or in short cDNA) which subsequently is copied to double stranded cDNA. After one round of linear amplification, the nucleic acids representing the original RNA composition is fluorescently labelled. Successful labelling is verified by measuring the labelling intensity of the amplified material. At this moment the labelled material can be applied to the actual microarray to allow for hybridisation (Fig. 6; middle panel). Inside the hedged box, hybridisation for
one probe set is visualised. A probe set contains DNA detection probes of one oligonucleotide sequence of 20 to 70 base pairs in length complementary to the gene of interest. Since each gene is represented by multiple probe sets to avoid non-specific detection, a complete array contains over a 100,000 probe sets to measure expression of all genes in the human genome. During hybridization, labelled RNA of a sample is deposited on a probe set only if it is complementary to the probe sequence. At the end of the hybridisation time using a high-resolution scanner, signal intensities for all probe sets are measured allowing the gene expression levels for each gene in a sample to be calculated. A typical microarray experiment generally involves dozens of tumour samples yielding gene expression data for all these samples at the end of the wet part of a study for data analysis (Fig. 6; bottom panel). Various statistical tools are currently available to identify genes that are at the mRNA expression level associated with the clinical endpoint of interest. For predictive markers the endpoints can be groups (patients who relapsed or not or patients who did or did not respond to a particular treatment) or time dependent measures (time to relapse and time to therapy failure). To identify genes that are differentially expressed between groups, e.g. responders and non-responders, a parametric t-test or equivalent is often used. However, due to the high dimensionality of gene expression data a correction to limit false discoveries is advised [16,17].

To predict classes, multi-gene predictors are developed from the list of differentially expressed genes. For time dependent measures, Cox regression and multivariate Cox models or equivalent are used for gene selection and endpoint prediction, respectively [18]. Apart from these down-to-earth outcome predictors, mathematically more complex algorithms such as principle component analysis [19], support vector machines [20], artificial neural networks [21] can also be used to build a classifier. Finally, a predictor is usually optimised for sensitivity or specificity depending on the clinical question and its performance is judged using leave-one-out cross validation. However, to truly test the performance of a predictor the use of independent cohort(s), set aside upfront or evaluated later on, has been proven to be essential. Finally, the results of a particular analysis are often visualised by hierarchical clustering [22] using the identified differentially expressed genes or using the selected set of prognostic or predictive genes. With regard to the clinical significance of gene expression profiling, the breakthrough came from the Stanford Laboratory who identified, based on global gene expression, five major subgroups in clinical breast cancer specimen [23].

Thus, within both the ER-positive (i.e. luminal A and B) and negative (i.e. basal and normal-like) breast cancers, at least two biologically quite distinct subgroups are present that are different with regards to aggressiveness [24,25] and response to neo-adjuvant paclitaxel containing chemotherapy [26]. After these first landmark observations, the field has rapidly progressed and gene signatures associated with endocrine therapy resistance [27], and chemotherapy response (Martens and colleagues, in preparation) [28–30] have been revealed and predictors of bone and lung relapse uncovered [31–33]. For clinical use, the most promising assays are gene expression signatures predicting disease recurrence in breast cancer patients with lymph-node negative disease. Starting with the 70-gene signature predicting disease outcome in young patients [34,35], a wound-healing signature [36], a Nottingham Prognostic Index [37] and genomic grade signature [38] predicting disease recurrence have all been identified. A robust 76-gene signature was developed for the prediction of distant metastasis in lymph-node negative patients irrespective of age and hormone receptor status [39]. In addition to this, in patients with lymph node-negative and oestrogen receptor-positive disease receiving adjuvant endocrine therapy with tamoxifen, a 21-gene recurrence score [40] and a 2-gene signature, the IL17BR-HOXB13 ratio [41] predicting disease recurrence have been identified. Of these signatures some have been validated [42,43] but only the 70-gene signature from Amsterdam and the 76-gene signature from Rotterdam have been validated in large independent multi-centric cohorts [44–46]. This has allowed the initiation of prospective evaluation of the performance of selected prognostic gene signatures in Europe and in the US. Furthermore, for a specific validated multi-gene classifier the FDA recently approved the technology to be used as a prognostic tool in the clinic. In conclusion, various predictive gene signatures have been identified while others are being developed. Even though most of them still need independent validation, it seems no dispute that predictive gene signatures are likely to be used for therapy decisions in the future.

Protein biomarkers

‘Proteomics’ is a rapidly developing area of cancer research which promises to have an enormous impact on prediction of therapy response in the individual patient. Gel-based and high throughput proteomic technologies, including two-dimensional gel electrophoresis, one- and two-dimensional liquid
chromatography, and proteomic microarrays in combination with mass spectrometry (MS) are currently the main tools available to mount a search for cancer protein biomarkers. Proteomic technologies are used for identification of new markers as well as for studying potential differences in the processing of already established protein markers. The hypothesis is that the protein biosynthesis machinery of cancer cells is significantly changed relative to normal cells in relation to the production, degradation and post-translational processing of proteins.

When the protein marker is identified, several different methodologies can be applied when validating the marker in clinical material. The more common methods include immunohistochemical staining of frozen or formalin fixed tissue, and tumour tissue protein extractions followed by immunological methods such as Enzyme-linked Immunosorbent Assay (ELISA) or Radio-Immunno Assay (RIA). The latter methods can also be applied on bodily fluids such as plasma, serum, urine or saliva.

At present a number of proteins, e.g. ER, PgR, ERBB2, and c-kit have proved their value in predicting clinical sensitivity/resistance to targeted cancer therapy. However, no protein measurement is in routine use for prediction of sensitivity/resistance to conventional cytotoxic chemotherapy.

Since most types of chemotherapy induces cell death by activating the apoptosis machinery in the cancer cells, many attempts have been made to relate amount of anti-apoptotic proteins in the cancer cells to degree of sensitivity/resistance to chemotherapy. We have chosen to report on one of these proteins, Tissue Inhibitor of Metalloproteinases type 1 (TIMP-1), since this protein has recently been shown to be involved in cellular protection against apoptosis and thereby resistance to chemotherapy [47]. The TIMP1 gene is located on chromosome Xp11.23−11.4 and codes for a soluble 28.5 kDa glycoprotein that consists of 184 amino acids in the mature form. The TIMP-1 protein can be present as a precursor form, as an unbound protein (free TIMP-1) or as a 1:1 stoichiometric complex with proMMP-9 or any of the non membrane-bound active matrix metalloproteinases (MMPs), in the latter case inhibiting the proteolytic activity of the enzymes (for review see Würtz and colleagues [48]).

TIMP-1’s inhibition of tissue remodelling processes, such as inflammation, wound healing, and cancer invasion, has mainly been ascribed to its inhibition of MMP mediated proteolytic activity. In contrast, the anti-apoptotic function of TIMP-1 has been shown to be MMP-independent. The MMP independent anti-apoptotic pathway was first demonstrated in a study of Burkitt’s lymphoma cell lines [49,50]. In this study, a positive correlation between TIMP-1 expression and resistance to apoptosis was shown. Addition of recombinant TIMP-1 resulted in inhibition of apoptosis, and addition of anti-TIMP-1 antibodies to neutralise secreted TIMP-1 resulted in a four-fold increase in induction of apoptosis. Of specific interest was that reduced or alkylated TIMP-1, completely devoid of all MMP inhibitory activity, effectively inhibited apoptosis in Burkitt’s lymphoma cells [49,50].

Recently, TIMP-1 was shown to interact with CD63 in MCF10A cells [51]. CD63 is a member of the tetraspanin family. Of particular interest was that interaction of TIMP-1 with CD63 inhibited caspase mediated apoptosis. Binding of TIMP-1 to the cell surface initiates a signal transduction cascade through Ras. Ras increases phosphorylation of ERK, and activates the Raf-1/tyrosine kinase/mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) signal pathways leading to stimulation of Cyclin D1 expression. By constitutive activation of focal adhesion kinase (FAK) and/or through the PI3 kinase, which phosphorylates Akt and Bad, TIMP-1 increases the expression of the anti-apoptotic protein Bcl-XL and thereby preventing activation of the caspase cascade [52]. Fig. 7 shows part of the intracellular signalling induced by TIMP-1 binding to CD63.

In cell culture based systems, lack of cancer cell TIMP-1 expression results in increased sensitivity towards chemotherapy [47], suggesting a potential use of TIMP-1 tumour tissue measurements in predicting sensitivity/resistance to chemotherapy in clinical cancer. Indeed, we have recently published that women with metastatic breast cancer and high tumour tissue
TIMP-1 values hardly show any objective response to antracycline based chemotherapy (0%) while patients with low TIMP-1 levels had an objective response rate of 45% [53].

Similarly, in a study including patients with metastatic colorectal cancer who received 5FU, leucovorin and irinotecan, high plasma TIMP-1 levels (dichotomised by the median plasma TIMP-1 value) were indicative of low probability of objective response to chemotherapy [54]. This decreased probability of obtaining an objective response to chemotherapy in plasma high TIMP-1 patients was reflected in a significantly decreased time to progression and overall survival of the patients with 62% of TIMP-1 low patients being alive at 24 months following treatment compared with only 7% of patients with high plasma TIMP-1 levels being alive at this time point. The Gastrointestinal Cancer Group and the PathoBiology Group of the European Organisation for Research and Treatment of Cancer are now in the process of initiating a prospective study validating these findings.

A recent publication [55] describes the association between plasma TIMP-1 levels and response to endocrine therapy in breast cancer. In a cohort of 251 patients with metastatic breast cancer treated with second line endocrine therapy, the objective response rate (CR+PR+SD) was 42% in patients with low TIMP-1 levels while the objective response rate in TIMP-1 high patients was only 16%. This difference was also reflected in a significant longer time to progression and a significant longer survival of plasma TIMP-1 low patients.

Using a gel-based method for a mass spectrometric site-specific glycoanalysis, we have recently characterised the glycosylation pattern of TIMP-1 in healthy plasma and platelets [56]. The glycoprofiling was performed using matrix-assisted laser desorption/ionisation MS and MS/MS. A total number of 38 glycopeptides were characterised. The glycans were of the complex type mainly comprising biantennary glycopeptides and diversified array of targeted assays based on highly integrated genomics and proteomics platforms. However, our understanding of how multiple genes and biochemical pathways interact in and between cancer cells is currently limited. Furthermore, despite the tremendous progress in the field, many important mechanisms involved in cancer progression and metastasis remain poorly understood or even unknown. These factors are the major reasons why so much emphasis is now being placed on functional assays that involve living malignant cells analysed ex vivo to assess therapeutic efficacy of anti-cancer therapies. In contrast to targeted assays performed on dead cells (e.g. immunohistochemistry and FISH) or extracted cell components (DNA, RNA, protein), functional assays integrate various components from individual cellular events, including tumour related pathways and mediators that are yet unknown and therefore cannot be evaluated through target specific testing. However, the one disadvantage of these assays is the need for live tumour cells, which in the case of metastatic disease could represent a limiting step. The underlying principles of ex vivo drug response testing were derived from in vitro assays for evaluating the activity of antibacterial agents pioneered by Robert Koch and Louis Pasteur in the late 19th century. In 1953, Black and Spear described the first modern ex vivo human tumour assay in which an attempt was made to predict clinical response to aminopterin in vivo on the basis of its cytotoxic activity in vitro [59]. This approach was further developed by Salmon’s [60] and Von Hoff’s [61] groups in the 1970–1980s. The next steps towards optimising this approach were to use a two-layer agar system to exclude non-malignant cells from the analysis and to utilise 3H-thymidine incorporation in tumour cell DNA as an assay endpoint [62]. The resulting test, referred to as the EDR assay will be discussed in this review as an example of a rationally designed ex vivo functional assay that can be used to predict high likelihood of chemotherapy resistance in various tumour types.

The EDR Assay is an in vitro drug resistance test that identifies with >99% accuracy patients that will not respond to a cancer therapeutic drug or drug regime [62]. This assay can thus be used to exclude agents that are unlikely to be clinically effective, thereby resulting in decreased side effects, improved response rates and prolonged survival of cancer patients [63–67]. The EDR assay utilises living
disaggregated tumour cells obtained from a cancer biopsy and exposes them in culture to chemotherapeuetic agents at suprapharmacologic concentrations using a tritiated thymidine endpoint (Fig. 8). Tritiated thymidine passes through the cell membrane and is converted in a stepwise manner to $^{3}$H-dTMP by thymidylate kinase and nucleoside diphosphate kinase. The tritiated dTMP is then incorporated into DNA during the S-phase of the cell cycle. Tumour cells affected by the anticancer drugs do not divide, or divide more slowly, and therefore incorporate less of the radioactive thymidine. By contrast, cells that are resistant to the drug continually divide and incorporate radioactive thymidine at a higher level.

It is important to emphasise that in the EDR assay biopsy derived living cells are cultured in 0.3% semi-liquid agarose on the top of the solid 1% agar underlayer, thus allowing only tumour cells to proliferate and excluding normal, non-malignant cells from the analysis.

By measuring the amount of radioactivity in a sample, the EDR assay determines the relative resistance of an individual patient’s cancer cells to a number of different anti-cancer therapies. An algorithm, based on Bayesian statistics, is then applied to determine the probability that a patient will respond to various therapies. It incorporates the percent of growth inhibition (PCI) caused by a particular drug and classifies the patients as having extreme drug resistance (EDR) if the PCI is greater than the median plus one standard deviation, or intermediate drug resistance (IDR) – between one standard deviation and the median, or low drug resistance (LDR) less than the population median. This algorithm is based on the median percent inhibition for a given chemotherapeutic agent across a population of tumour specimens (currently, >125,000 cases in the Oncotech database (personal communication)).

The EDR Assay is highly accurate at predicting clinically inactive drugs and patients whose cancer cells have Extreme Drug Resistance, with <1% response rate to the given agent [62]. Patients with Intermediate Drug Resistance have a finite, but less than average, likelihood of responding to a drug. No definitive conclusions can be drawn for therapeutic advantage in the activity of agents to which a tumour has Low Drug Resistance [68]. Fig. 9, which is derived from the initial clinical study validating the EDR assay [62], shows 450 double-blinded correlations between clinical response to chemotherapy (non-responders versus responders) plotted against individual data generated in the EDR assay (expressed as PCI). While 52% (115 out of 222) cases in the LDR group responded to chemotherapy, only 0.8% (1 out of 127) of patients in the EDR category achieved detectable clinical responses.

Drug exposures in the EDR assay are greater than those achievable pharmacologically. The period of drug exposure is significantly longer (days in the EDR Assay, as compared to hours in the patient) and at concentrations which continuously approximate the peak plasma level. As an example, for Paclitaxel, this would translate to an 8.1-fold greater exposure in the EDR assay than what is routinely achieved at standard doses in the patient. When tumour cells continue to proliferate in the presence of drug at such
levels, it is unlikely that the tumour will respond to the significantly lower exposures achieved in the patient [62, 67, 69].

The EDR assay can also be used to evaluate the activity of novel chemical entities being developed as anti-cancer drugs. New compounds can be tested in the EDR assay in the surrogate testing mode, to approximate a clinical EDR assay. After applying the algorithm to the tumours tested, one can stratify sensitive and resistant phenotypes within a single type of tumour or between tumour types. The sensitive and resistant tumours can then be compared through molecular or cellular analyses to identify biomarkers that are predictive of response. In addition, the EDR assay can detect tumour types, which are innately resistant to the compound. This information can be subsequently used to choose tumour types or a subset of tumours in which the compound will be more active in the patient, and, thus, be more likely to get regulatory approval.

Several studies performed by Oncotech and independent research groups in ovarian, breast, and brain cancer, as well as some recent studies in other tumour types (colorectal, lung, and endometrial cancers; melanoma) indicate that lack of in vitro cytotoxic activity of several chemotherapeutic agents measured in the EDR assay is associated with poor patient outcomes [64, 70, 71]. In a prospective EDR assay-guided study on 100 patients with recurrent ovarian cancer, clinical response to platinum based regimens was compared in 50 women whose chemotherapy was based on the results of the EDR assay versus clinical response in 50 control cases treated empirically [72]. This study demonstrated that platinum-sensitive patients in the EDR assay-guided group exhibited higher overall response rate than those in the control group (65\% versus 35\%, respectively; \(P = 0.02\)). Furthermore, as shown in Fig. 10, platinum-sensitive patients in the EDR-treated cohort had longer overall and progression-free survival rates (38 and 15 months, respectively) than control, empirically-treated patients (21 and 7 months, respectively; \(P = 0.005\) for overall and \(P = 0.0002\) for progression-free survival).

However, despite the proven value of the EDR assay in predicting disease-specific survival and clinical resistance to chemotherapy in solid tumours (mostly, in ovarian cancer), the ability of the EDR assay to optimise specific clinical outcomes through assay-guided treatment decisions remains to be established in other tumour types. Supplementary data, especially from appropriately controlled prospective studies are needed to conclusively prove the value of the EDR assay in guiding chemotherapy in solid tumours and
its ability to improve objective clinical outcomes for cancer patients.

Clinical validation of predictive markers

In order to have clinical impact, a new predictive marker has to pass a number of analytical and clinical validation steps [73].

A major dilemma associated with assays for biomarkers is that various assays may employ different reagents (e.g. antibodies), which may generate non-equivalent test results. Also, variations in reagent preparations, sample processing and the use of different standards may result in discordant test results. Therefore, reagents, assays and procedures should be standardised and the quality of biomarker assay results should be monitored by continuous between-laboratory proficiency testing of performance. Biomarkers are often used in the clinical setting to provide additional information that will influence clinical decision making, while only few guidelines have been established to inform about how a biomarker should become standard for a certain type of cancer. Hayes and colleagues, 1996 [74] therefore proposed that it is highly necessary to establish standard criteria for evaluation of biomarkers and to standardise the biomarker information for clinical utility. To judge whether these factors have added value over the traditional factors, McGuire and colleagues, 1992 [75] proposed guidelines for evaluating new cancer biomarkers, including a biologic hypothesis for the new factor, adequate sample size, risk of sampling bias, appropriate test system, establishment of cut-off values in a training data-set and confirmation of the observation in a validation data set. Biomarker assay results are often quite heterogeneous, depending on the composition of the specimen, way of tissue processing, and design and specificity of an assay and, as important, statistical methods used for evaluation of data. It is of utmost importance to note that uniform handling applies to all of the laboratory steps including use of highly standardised and optimised reagents, tissue collection, storage and processing, the analytical procedures, and subsequent data processing. Within Europe, a multitude of translational multi-centre cancer studies have been co-ordinated by the EORTC. Within this consortium the PathoBiology Group was established to research and advise on common, or equivalent, methodologies for biomarker assays and to ensure that appropriate External Quality Assessment (EQA) schemes are applied. As an example, for the past 25 years for ER and PgR, large-scale EQA trials, amounting to participating 165 institutions/hospitals from 18 countries, have been carried out and organised by the PathoBiology Group [76,77].

When a validated assay is available the clinical studies can be initiated. It is recommended to start analysing material collected from prior well-controlled clinical studies, from which high quality sample material, as well as clinical outcome regarding treatment efficacy, is available. Following these retrospective studies, the prospective clinical studies can be started. We recommend that the first study is designed as an adjunct to a clinical study in which the primary objective is to test the efficacy of one or more drugs. In such a study, sample collection should be prospective and strictly follow predefined conditions (Standard Operating Procedures) regarding sample collection, storage handling, analyses and data reporting. The next step in the validation is a well-dimensional prospective study where the primary objective is to validate the predictive power of the marker in question. A simple trial design is as follows: Patients are randomised to receive either standard treatment or marker-guided treatment. End-points will be objective response rate (RECIST criteria), or time to progression and less frequent overall survival. These types of clinical studies can only be performed in cancer types for which more than one treatment option exists and are especially helpful if the efficacy of the different treatments is considered equal.

For example, in the majority of patients with metastatic colorectal cancer (mCRC), chemotherapy is the treatment of choice. Survival following chemotherapy of mCRC has improved substantially over recent years. Whereas survival without chemotherapy was limited to 8.5 months, the first effective drug (5-fluorouracil, 5FU) was shown to increase the median overall survival to 12 months [78]. Addition to 5FU of either irinotecan or oxaliplatin increased the efficacy: If one of these drugs is added to 5FU, the response rates rise from approximately 20% to approximately 50% with either the combination irinotecan/5FU (FOLFIRI) [79–81] or oxaliplatin/5FU (FOLFOX) [82–84]. Irinotecan and oxaliplatin based chemotherapy differs in toxicity profile (diarrhoea and alopecia versus neurotoxicity), but there is no meaningful difference with regard to the overall efficacy. Because efficacy and overall toxicity are similar in the whole patient population, both regimens are equally used in first line therapy.

One unresolved problem with either of the chemotherapy combinations is that up to 25% of patients have tumours that are inherently resistant to the chosen
chemotherapy schedule (FOLFOX or FOLFIRI) [81, 82,85]. The current clinical approach selects one treatment regimen over another based on predicted success (and to a lesser degree also toxicity) in large patient cohorts. Using this approach, there is, necessarily, a large fraction of individual patients who have no benefit whatsoever and may even suffer from the chemotherapy. These patients are unnecessarily exposed to treatment toxicity, and they experience disease progression, which affects the performance status and the capability to tolerate further chemotherapy. In contrast, they might rather profit from an alternative regimen.

In contrast to predict chemotherapy response, it is at present more important to predict chemotherapy resistant metastatic colorectal cancer. If chemotherapy resistance to one type of cytotoxic drug could be predicted for individual patients, these patients could already, as 1st line treatment, receive the other treatment combination and thereby increase their chance of treatment benefit. In addition, such an approach would avoid the costs for approximately 2 months of ineffective treatment (with weekly costs of up to €500), and thereby be highly cost-effective. With the currently available evidence, there is no way to predict response or resistance to the given chemotherapeutic treatment in the individual patient, let alone any guidance to select one regimen instead of another one.

Thus, there is a need for research focusing on the identification and development, including clinical implementation, of predictive markers for each existing chemotherapeutic drug in addition to general markers for chemotherapy sensitivity/resistance. Also, a clear discrimination between diagnostic, prognostic and predictive markers is needed, including statistical methods that can facilitate this separation.

Conflict of interest statement

Nils Brünner is an advisor to Oncotech Inc. The University of Copenhagen is holding patent applications on TIMP-1 as a predictive marker in cancer. Kirsten Vang Nielsen is an employer of Dako A/S. Eugene Mechetner is an employer of Oncotech Inc.

References

Biomarkers for therapeutic efficacy


