Designing personalised cancer treatments

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Abstract

The concept of personalized medicine for cancer is not new. It arguably began with the attempts by Salmon and Hamburger to produce a viable cellular chemosensitivity assay in the 1970s, and continues to this day. While clonogenic assays soon fell out of favour due to their high failure rate, other cellular assays fared better and although they have not entered widespread clinical practice, they have proved to be very useful research tools. For instance, the ATP-based chemosensitivity assay was developed in the early 1990s and is highly standardised. It has proved useful for evaluating new drugs and combinations, and in recent years has been used to understand the molecular basis of drug resistance and sensitivity to anti-cancer drugs.

Recent developments allow unparalleled genotyping and phenotyping of tumours, providing a plethora of targets for the development of new cancer treatments. However, validation of such targets and new agents to permit translation to the clinic remains difficult. There has been one major disappointment in that cell lines, though useful, do not often reflect the behaviour of their parent cancers with sufficient fidelity to be useful. Low passage cell lines – either in culture or xenografts are being used to overcome some of these issues, but have several problems of their own. Primary cell culture remains useful, but large tumours are likely to receive neo-adjuvant treatment before removal and that limits the tumour types that can be studied. The development of new treatments remains difficult and prediction of the clinical efficacy of new treatments from pre-clinical data is as hard as ever. One lesson has certainly been that one cannot buck the biology – and that understanding the genome alone is not sufficient to guarantee success. Nowhere has this been more evident than in the development of EGFR inhibitors. Despite overexpression of EGFR by many tumour types, only those with activating EGFR mutations and an inability to circumvent EGFR blockade have proved susceptible to treatment.

The challenge is how to use advanced molecular understanding with limited cellular assay information to improve both drug development and the design of companion diagnostics to guide their use. This has the capacity to remove much of the guesswork from the process and should improve success rates.

Keywords

ATP, cancer, genotyping, RT-PCR, cell, culture

Introduction

The goal of truly personalised cancer treatment is within our grasp. It is no longer acceptable to suggest one size fits all treatment to patients who understand that their cancer is unique to them, as indeed it is. Treatment based on randomised clinical trials has improved cancer outcomes, but has done this incrementally. Standard therapy inevitably fails many patients as it ignores the heterogeneity of tumour response to drugs (fig 1). Richard Klauser decribed the situation at the turn of the century very well in 1997 when he said, "Right now we lump patients together and treat them with the same drugs and then deal with their variable response to treatment. We're essentially treating different diseases with the same medicine." That cannot be good medicine, and recent gains made suggest that individualised therapy based on companion diagnostics can do better. The problem of course is knowing who has which disease, or at least who will benefit from which drug. Oncologists need pathologists to do a test and tell them which drugs are likely to work. They can then decide on the appropriate treatment and exercise the real skill of getting the best from the drugs in patients despite variable side effect profiles.

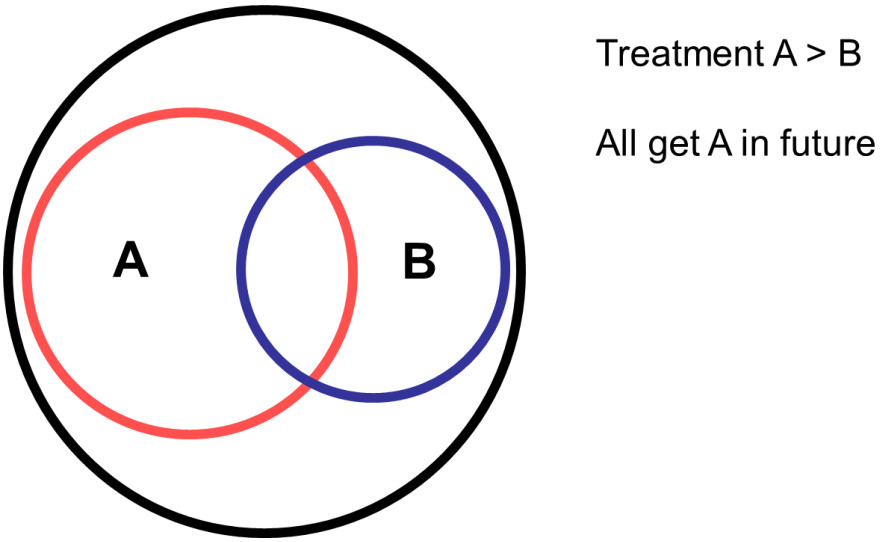

Figure 1. This Venn diagram shows the hypothetical result of a standard clinical trial of two treatments – A and B. Treatment A does better than B, and becomes the new standard of care. However, those outside the circles representing patients responsive to either treatment get the toxicity and no benefit, while those within B but not A, would have responded to an older treatment which they will not even know they could have benefitted them.

When considering the sort of test to use, it is important to distinguish between prediction and prognosis. A test that defines prognosis simply implies that two (or more) groups are different and that you they can be identified by the test. However, the test does nothing to improve the prognosis this contrast to a predictive test which alters the outcome in one group resulting in improved prognosis or for some of the patients (Figure 2). Some tests do both – for instance the Herceptest which defines a group of patients who do worse if not treated, and predicts that they will do better if treated with an anti-HER2 antibody, traztuzumab (Herceptin).

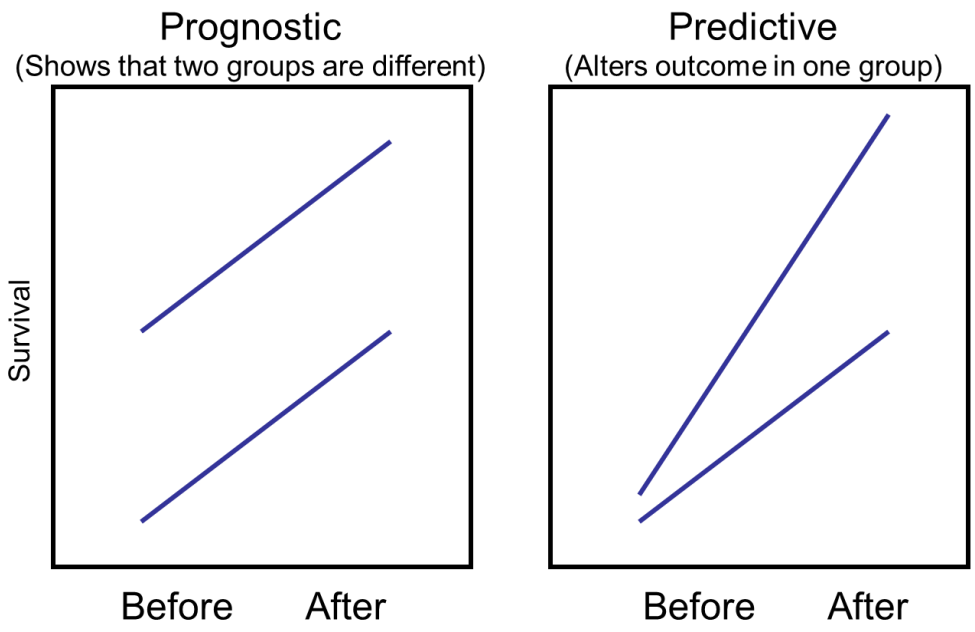

Figure 2. The difference between a prognostic and a predictive test. Prognostic assays distinguish groups according to their risk of an adverse outcome, while predictive tests define a group in which treatment will improve outcome.

Cellular cytotoxicity assays

There have been many attempts to individualise cancer therapy using tests based on exposure of cells to drugs. None of these have entered clinical practice, but many have provided very useful research tools for the development of new drugs and have increased the understanding of anti-cancer mechanisms. The field started in the 1970s with clonogenic assays pioneered by Salmon and Hamburger (1). These proved relatively difficult to handle with a high failure rate as many patients' tumours could not be grown in vitro. Many other assays have been produced since(2). The enzyme release assays look for cell death by release of enzymes into the medium surrounding the cells. Viability assays such as the trypan blue assay are in regular use by labs growing cells as they are simplicity itself. They show that the cells have intact membranes and recent automation of these methods has extended their utility. The NCI60 panel has made extensive use the Sulforhodamine B (SRB) assay which determines the total protein content of the culture compared with controls. There are a number of cell death assays on the market which are particularly useful for looking at apoptosis and other cell death mechanisms. They include the caspase assay, the Annexin V assay, and various methods to assess DNA fragmentation. Cell survival assay are probably the most widely used. The Neutral Red uptake assay was the first such test, but was rapidly supplanted by the widely used MTT assay which relies on succinate dehydrogenase activity and hence intact mitochondrial function. The ATP Tumour Chemosensitivty Assay (ATP-TCA) is an example of cell survival assay and is covered in greater detail below. All of these assays have their advantages and disadvantages and it is a case of choosing the correct test for the job.

The ATP-TCA

The ATP-based Tumour Chemosensitivity Assay (ATP-TCA) was developed by Peter Andreotti and the author in the early 1990s as a standardised way of assessing the effect of anticancer drugs in primary cell cultures (3, 4). It uses fragments of tumour from fresh tumour resection specimens or biopsies obtained directly from the operating theatre. The fragments are minced, and incubated overnight with a relatively gentle collagenase based medium resulting in a suspension of single cells and small clumps which can be washed, counted and then plated easily at 20,000 cells per well in a 96 well polypropylene plate. Drugs are added and the plates of incubated for six days at 37 C in a CO2 incubator with high humidity. At the end of that time, the cells are extracted and ATP content measured by luciferin-luciferase. In each 96 well plate it is possible to look at four different drugs or combinations at six dilutions, using triplicate wells to ensure accuracy. One row within the plate is reserved for a medium only control and the other for a maximum inhibitor which should kill all the eukaryotic cells present and shows the baseline luminescence read by the luminometer. In common with most such assays, the results are then expressed as the percentage inhibition against a test drug concentration.

The test drug concentration used in such tests can be hard to determine, as the concentration of drug to which the tumour is exposed may differ from the free drug concentration in blood and there is usually reduced protein binding in tissue culture media. Data from manufacturers and phase I trial reports contain most of the information needed. For the ATP-TCA, we were able to come up with a test drug concentration for most cytotoxic drugs, allowing simple comparison of the effect of individual drugs against tumours. The other issue is drug metabolism: drugs such cyclofosfamide require activation by the liver, but in this and some other cases, it was possible to obtain metabolites for use in vitro.

The ATP-TCA proved to be a very useful test for drug development, allowing early testing of compounds against different cancer types and giving some indication of likely activity. This included cytotoxic agents, anti-tumour antibodies and targeted small molecules (5-15). The assay is particularly helpful for the in vitro design of new combinations, particularly where there was a molecular hypothesis to test. For instance, we were able to show that the effect of mevalonate pathway inhibitors was limited, but that they enhanced the effect of N-bisphosphonates, probably via the production of metabolites not normally present in the cell (11). We have most recently used the assay to show that EGFR and PI3K blockade is synergistic in ovarian cancer cells (Glaysher et al., unpublished data).

One important use of the assay was to show the level of heterogeneity present between patients in terms of their response to new and old drugs. We were able to do this for a variety of tumour types, and this suggested that the results might be useful to guide treatment. We undertook the first randomised clinical trial of individualised chemotherapy and were able to show impressive progression free survival and response rates in the assay-directed arm of the study in recurrent platinum-resistant ovarian cancer, though the size of the trial was limited and the effect just fell short of statistical significance. There was, however, a statistically significant benefit to patients when the oncologists switched their preference to one of the combinations developed using the assay, rather than standard treatment (16). Furthermore third line treatment of patients in the physician's choice group resulted in a 40% response rate, unheard of in such cases.

Primary cell culture

One feature of primary cell culture is that it recapitulates the biology of tumours far better than cell lines. Whereas cell lines are grown in growth supporting media and have high S-phase fractions, this is not true of most solid tumours, even the most proliferative of which rarely achieve more than 50%. The

reason for this difference is largely due to the presence of fetal bovine serum within the culture medium and the use of plastics that allow cell adherence. The medium used in the ATP-TCA does not suffer from this particular problem as it does not contain serum and the assay uses polypropylene plates so that the cells effectively grow within suspension. We were able to show in experiments with cell lines that if these were cultured without serum under adherence-free conditions, then those that survived recapitulated the chemosensitivity of the parent lines much better. Using this approach we developed a 20 cell line panel suitable for high throughput testing (17). The alternative approach would be to use low passage cell lines or xenografts of human tumours.

One major problem of primary cell culture is its requirement for large numbers of fresh tumour cells, which have to be obtained from surgical specimens. Due to advances in screening and neo-adjuvant therapy, many tumours are now of insufficient size to allow large amounts of tissue to be taken for research. There are still a few exceptions – notably ovarian cancer. In an effort to meet this need, we have developed automation of the AT P-TCA using a 384 well robot (18). We were able to show that the chemosensitivity of cells in either format is similar and then went on to test the library of over 5000 plant extracts against three different varying ascites in just three weeks. The results allow us to find around 20 hits for further investigation. These hits were not found using cell line screens.

The ATP-TCA and similar assays offer a number of advantages. They assess all pharmacodynamic mechanisms at once, provide tumour derived cells to test new drugs, allow the design of new combinations, and allow mechanistic studies of resistance and sensitivity to new or old agents. They can also provide relatively simple data to the oncologist and have an extensive publication record. Their major disadvantage is that they need fresh tumour tissue, and a lot of it. This usually necessitates a change of clinical practice and needs expert staff on hand with a suitable laboratory. None of these assays examine pharmacokinetic variables, which can of course be a very important of response.

Molecular methods

The use of molecular methods to obtain sensitivity data from tumour tissue is increasingly possible. DNA or RNA can be extracted simply and effectively from formalin-fixed paraffin embedded (FFPE) tissue blocks stored in the pathology department following diagnosis. It is possible to go back at least a decade to obtain material. Sequencing can then be used to define the genetics of individual tumours, while quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) can be used to define gene expression with impressive accuracy. Since most drugs hit proteins, their expression and function may need to be assessed directly. Protein expression is probably best assessed by immunohistochemistry which can be improved by the use of careful external quality assurance, standardisation, and automation. Digital pathology is now coming into practice and is doing for pathology what digital archives did for radiology. Any of these techniques can be used as companion diagnostics. However, the choice of method for companion diagnostics is important as it will impact on the ease with which patients can be treated and the costs within the laboratory.

Hanahan and Weinberg describe the hallmarks of cancer and their latest revision (19) suggested that there was potential for inhibition of any of these hallmarks by therapeutic agents. The intelligent use of these agents in alone and in combination requires considerable understanding of the biology behind the individual tumours under treatment (not just their histological types). We have recently investigated the use of sequencing using the IonTorrent PGM (Life Technologies, Glasgow, UK) for better genetic classification of tumours, together with a network of eight laboratories across Europe. The initial results

for this 22 gene panel which assesses 504 mutation hotspots are excellent and suggest that sequencing is close to becoming a routine technique in clinical laboratories. Simpler PCR based methods (fig 3) also have the ability to define patients with EGFR or KRAS mutations, but there is a tipping point, where if one needs more than 2-3 mutation assays per patient, it is actually less expensive to sequence the genes of interest.

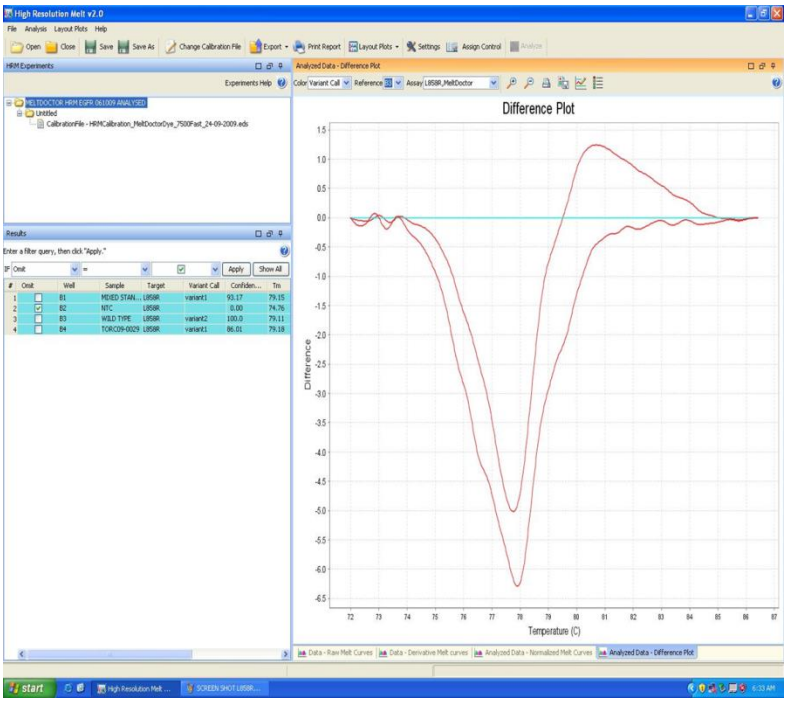

Figure 3. Simpler PCR based methods such as melt curve analysis can be an effective and inexpensive way of showing the presence of mutations, in this case an EGFR mutation in a patient with non-small cell lung cancer.

The molecular pathology of individual genotypes is complex and we are far from being able to treat every patient. There are lessons to learn from examples such as the use of EGFR inhibitors in lung and colorectal cancer. The EGFR signal transduction pathway is the same, but the cell's reliance on it varies between these two tumour types. Lung cancers with activating mutations of EGFR respond well to small molecular inhibitors of the EGFR ATP binding site. In colorectal cancer there are no EGFR mutations, but KRAS mutations are common. Patients with these mutations do not respond to EGFR blockade by EGFR antibody, but those without KRAS mutations do, though small molecule inhibitors of EGFR are ineffective.

Mechanisms of anticancer drug resistance

The development of resistance to anticancer drugs is of considerable importance to patient outcomes. Using the ATP-TCA we showed that breast cancer could develop resistance rapidly in up to 2/3 of patients treated with combination chemotherapy (20), and later showed that this was due to the up-regulation and down-regulation of gene expression (21) rather than clonal evolution as was commonly

thought at the time. Despite the undoubted advantages of the newer targeted agents over most older cytotoxic drugs, the generic determinants of resistance and sensitivity to anticancer agents (table 1) remain important in defining patient response to targeted agents.

Table 1: The determinants of anti-cancer drug resistance

- Alteration of drug targets
- Expression of drug pumps
- Expression of detoxification mechanisms
- Reduced susceptibility to apoptosis
- Increased ability to repair DNA damage
- Altered proliferation

Sazaki et al. recently published a number of interesting methods by which tumour cells have outwitted crizotinib and become resistant despite having an ALK translocation (22). In one case the tumour had a new mutation within ALK, while in another concurrent EGFR and ALK translocation led to complete resistance. There is therefore a need to try new targeted agents within appropriate cell based models, and these can be helpful in defining resistance mechanisms.

Changes in gene expression can be assessed by qRT-pCR. We have used the Taqman Array (Life Technologies) to assess the expression of 92 genes implicated in resistance to anticancer agents by qRT-PCR in a number of tumour types (23-25). This required just two 1 mm punches from FFPE tumour blocks. The results are remarkable. In multiple linear regression models of 5-15 genes, there was excellent correlation between chemosensitivity of tumour cells in the ATP-TCA and gene expression for the majority of drugs, as illustrated for breast cancer in figure 4. This was also true of combinations, suggesting that the method could be applied in the clinic.

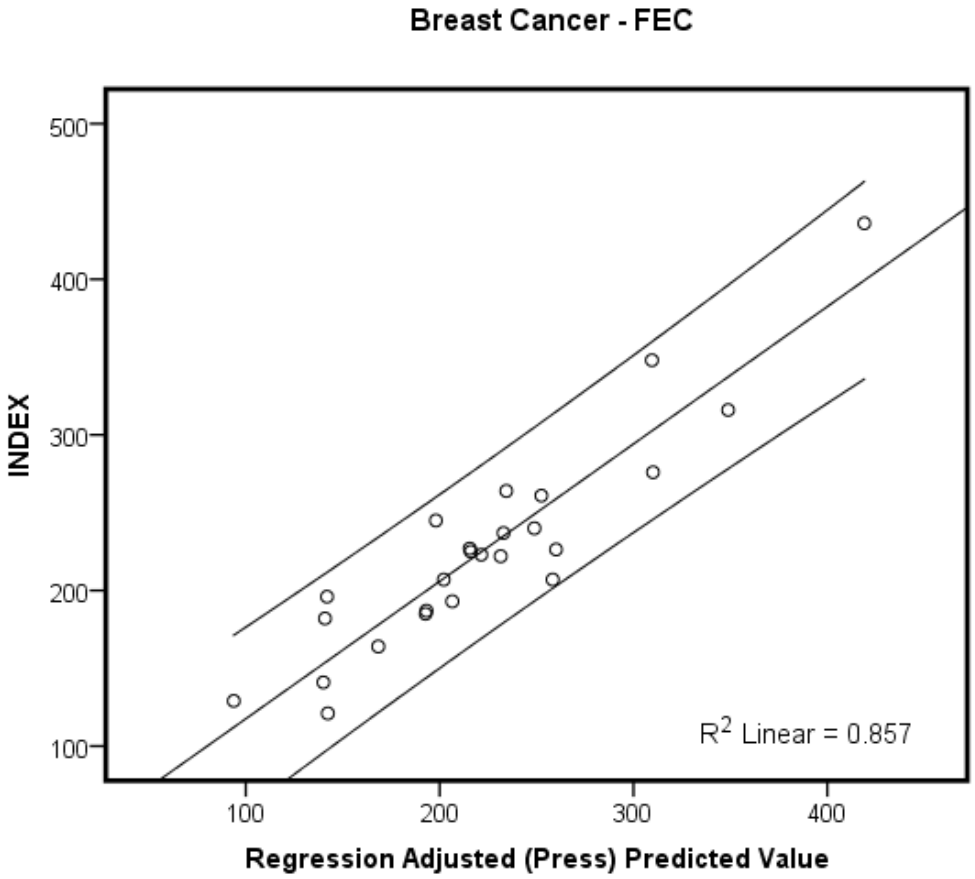

Figure 4. Comparison of ATP-TCA index results with predicted index from gene expression in a series of 25 breast cancers exposed to FEC (5-fluorouracil, epirubicin, and cyclofosfamide) in primary cell culture. The adjusted correlation coefficient is 0.86 for a model using data from just 12 genes.

Conclusions

Cell line and primary cell cultures both have a role in drug development. The use of primary cell culture permits the molecular investigation of sensitivity and resistance in a way that is difficult to achieve from clinical data, and can predict the response to patients to anti-cancer agents. It is feasible to use molecular data to predict the sensitivity of cancer cells to new agents, provided that there is sufficient understanding of the biological pathways involved. The challenge now is to use advanced molecular understanding with limited cellular assay information to improve both drug development and the design of companion diagnostics to guide their use. This approach has the capacity to remove much of the guesswork from the process and should improve success rates.

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