expert reviews
in molecular medicine

Clinical relevance of the molecular mechanisms of resistance to anti-cancer drugs

Matthew Links and Robert Brown

Resistance to anti-cancer drugs (drug resistance) can be defined in the laboratory by the amount of anti-cancer drug that is required to produce a given level of cell death (drug response). Clinical drug resistance can be defined either as a lack of reduction of the size of a tumour following chemotherapy or as the occurrence of clinical relapse after an initial 'positive' response to anti-tumour treatment. Many studies of tumour samples do not directly measure drug resistance in the laboratory (because it is difficult to perform functional assays on tumour tissue); instead, key proteins or genes that are involved in particular mechanisms of drug resistance have been proposed as 'markers' of drug resistance. In this review, we have focused on the problems that can arise when attempts are made to relate the relevance of laboratory-identified molecular mechanisms of drug resistance to anti-cancer drug resistance that occurs in patients.

Drug resistance can occur at multiple points between the administration of the drug to the patient and the desired effect of tumour-cell death. These include: (1) changes in the metabolism of the drug, (2) penetration into the tumour microenvironment, (3) intracellular uptake, followed by (4) interactions with the target and (5) subsequent signalling events. The combination of information about all of these variables represents a major challenge.

One aim of research into drug resistance is the development of (prognostic) models that allow the prediction of clinical response; however, progress in this area is hampered by the complexity, redundancy and interdependence of the biological systems that are involved in drug responses. Redundancy (the presence of proteins or pathways with overlapping functions) minimises the significance of a particular pathway. Interdependence (the occurrence of interactions or 'cross-talk' between pathways) complicates the interpretation of changes in a single pathway because of the potential for this to be the result of (or the cause of) changes in other pathways.

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For many resistance markers, variability in both the methods used and the patient populations studied has contributed to conflicting results being reported in the literature. These problems of variability have been discussed, together with some of the statistical issues that are associated with the evaluation of many determinants of drug response. Strategies have also been suggested to minimise between-study variability and to summarise input from both redundant and interdependent systems.

A second aim of drug-resistance research is the development of new strategies to overcome drug resistance, such as the development of drugs that overcome or modulate particular resistance mechanisms. The evaluation of such compounds in clinical trials poses particular difficulties, and the contribution of validated intermediate endpoints, which measure the desired biological effect(s), has been highlighted.

Finally, advances in (1) genome-wide screening for markers of drug resistance and (2) rational (targeted) drug design, and the relationship of both of these to the molecular analysis of tumours have been described. The potential that these developments offer to improve the selection of those patients who are most likely to benefit from existing therapies, and also to the development of new approaches to overcome drug resistance in cancer have been emphasised.

Drug resistance (i.e. the failure of tumours to respond to chemotherapy or the occurrence of relapse with disease, which is resistant to further treatment, after an initial response) is the major limitation to the effectiveness of current cytotoxic cancer treatment regimens. There are two main strategies for improving this situation: one is to develop new treatments, and the other is to apply existing therapies more effectively. The study of the molecular mechanisms of drug resistance should facilitate both strategies, allowing both the development of novel drugs (or combinations of therapies) to circumvent drug resistance and also the more rational selection of existing therapies for defined groups of patients.

Molecular markers

Clinical prognostic factors predict poorly the response of a tumour to particular drugs. However, there is an ever-growing list of molecular markers of drug resistance that have been shown in the laboratory to have a role in mediating the response of tumour cells to chemotherapy. The ultimate aims of studying such molecular markers of drug resistance are to: (1) select which patients will benefit from chemotherapy, (2) select which drugs to use and (3) develop therapeutic strategies for overcoming drug resistance. This would eventually allow treatment regimens to be specifically tailored to the molecular pathology of the tumour (Fig. 1).

The application of our increasing understanding of the molecular mechanisms of drug resistance to further these clinical aims has, thus far, been disappointingly slow. There are, however, some indications that information about molecular markers could soon be used in a clinical setting. The recent confirmation of important roles, in some specific contexts, for two molecular markers that have a long history of investigation, namely the proto-oncogene *ERBB2* (which is also known as *HER-2* and *Neu*; see Table 1) and the drug transporter multi-drug-resistance 1 (MDR1) protein (see Table 2), is encouraging.

The evaluation of existing molecular markers has revealed substantial difficulties in assessing the significance of potential predictive markers of resistance to cytotoxic drugs. A summary of the process that is required to validate a potential marker is shown in Figure 2. Some of the relevant issues and guidelines for such studies have been published by McGuire (Ref. 1) and Gasparini and colleagues (Ref. 2). Key points include: (1) the presence of a plausible biological hypothesis, which distinguishes between initial exploratory studies and confirmatory studies, (2) the need to consider the role(s) of the new predictive marker relative to those of existing markers and (3) the validation of the results in a prospective clinical trial. Unfortunately, there are, so far, very few examples of this process having been successfully concluded.

In this review, we have described some of the important issues and difficulties that are associated with the demonstration that a drug-resistance mechanism that has been identified in the laboratory is actually important in cancer patients; we have also suggested some strategies for overcoming these problems, and discussed some exciting new opportunities.

Determinants of drug response

Drug resistance can occur at multiple stages between the administration of a drug to a patient and the desired response, that is death of the tumour cells. Many factors aid or inhibit

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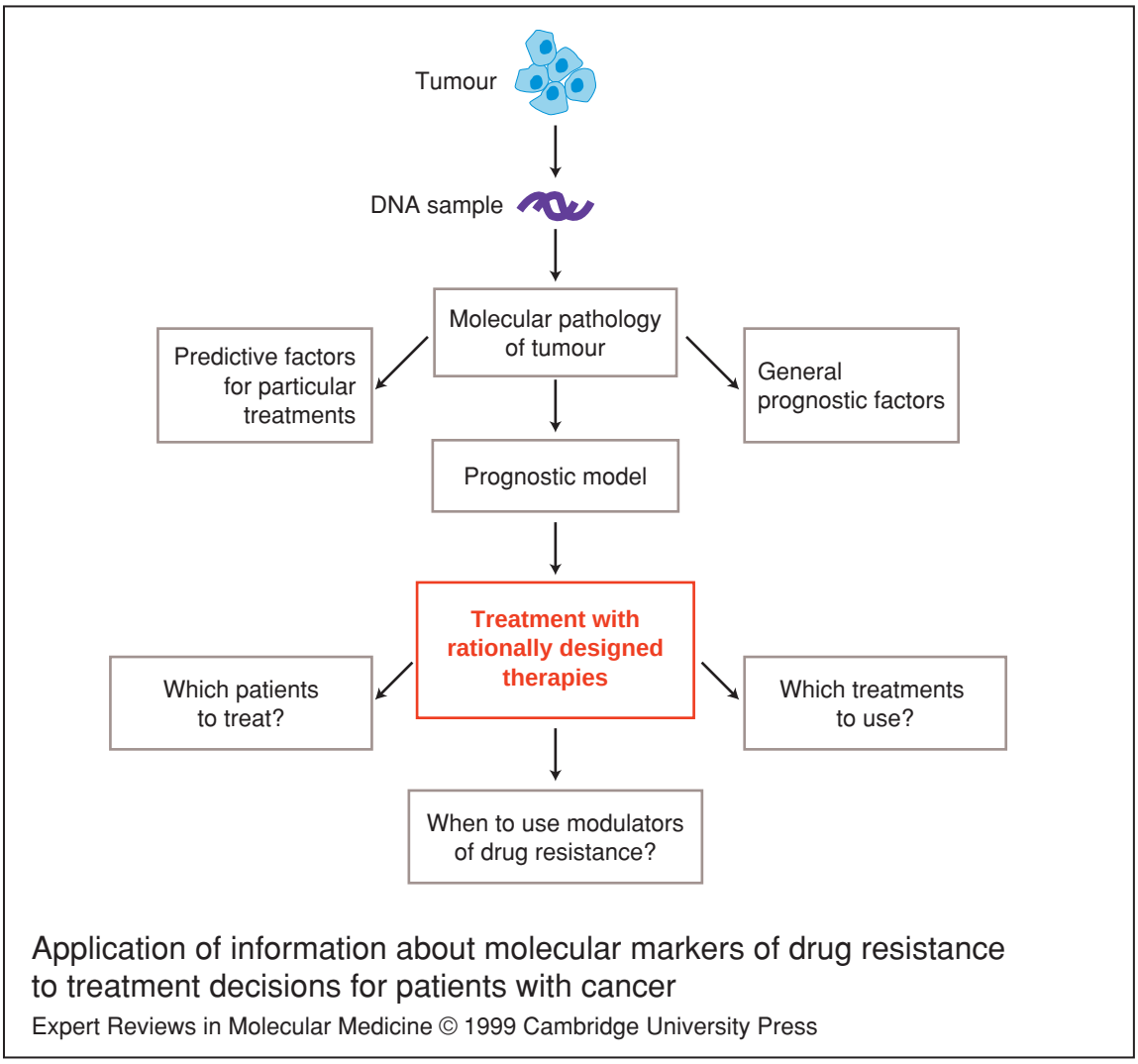

Figure 1. Application of information about molecular markers of drug resistance to treatment decisions for patients with cancer. DNA derived from tumours can be used to measure tumour-specific molecular changes and to determine the 'molecular pathology' of the tumour. Information from prognostic factors (which influence patient survival regardless of treatment) and from predictive factors (which influence the benefit of particular treatments) can be combined in a prognostic model to determine the likely survival of a patient with or without specific treatments. This information can then be used to determine which patients might benefit from treatment, which treatments to use and when modulators of specific drug-resistance mechanisms might be useful. The end result is a treatment plan that is tailored to the molecular pathology of the tumour of individual patients **(fig001rbg).**

the response of a tumour to a drug (its drug response). Drug resistance can occur because of biological variability in any of these factors and can occur through multiple mechanisms (see Fig. 3; Table 3). Further information about specific drug-resistance mechanisms can be found in the box entitled 'Further reading, resources and contacts'.

An artefact of the varying interests of individual investigators is that specific studies have tended to focus on one of three areas: (1) the pharmacokinetics of cytotoxic drugs (i.e. how a drug is 'handled' by the body), (2) intracellular drug transport (i.e. how a drug is taken into and exported out of cells) or (3) intracellular determinants of a drug response (i.e. target modification, drug inactivation and signalling pathways for apoptosis). Some issues such as variability in drug delivery from the plasma to the tumour microenvironment (Ref. 3) have been

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Table 1. ERBB2 and its potential as a marker of drug resistance (tab001rbg)

ERBB2 protein: ERBB2 [protein tyrosine kinase erb B2 precursor (also known as c-erbB-2 protein precursor or kinase-related transforming protein erbB2)] is a proto-oncogene that encodes a membrane-bound receptor tyrosine kinase of the epithelial growth factor receptor (EGFR) family. It is overexpressed in some breast, ovarian and colorectal cancers (Ref. 31). ERBB2 has a possible role in tumour-cell proliferation, tumour invasion and tumour metastasis and drug resistance.

Relevant drugs: The range of relevant drugs is unclear; however, the best evidence is provided by clinical data demonstrating resistance to cyclophosphamide, methotrexate and fluorouracil combinations.

Pre-clinical data: Limited evidence suggests that the overexpression of ERBB2 protein directly mediates drug resistance in cell culture; however, the mechanism by which mediation might occur is unclear.

Clinical data: There is evidence that the overexpression of ERBB2 protein is associated with a poor prognosis because of its effects on tumour proliferation and metastasis, although the effect is weak in node-negative patients. There is increasing evidence that the overexpression of ERBB2 protein is associated with a poor response to chemotherapy; however, this poor response can be overcome by modifying the chemotherapy treatment to include an anthracycline (Ref. 32). This counteractive effect has been confirmed in independent studies (Ref. 33).

Conclusion: These studies of ERBB2 have provided the first data to support the use of a predictive molecular marker to optimise the choice of chemotherapy regimens. Issues regarding the standardisation of test results and application of these data to treatment with different drugs and in other clinical settings require clarification.

Table 2. The multi-drug resistance 1 (MDR1) protein and the multi-drug-resistance (MDR) phenotype (tab002rbg)

MDR1 protein: An energy-dependent drug efflux pump that is responsible for decreased drug accumulation in multi-drug-resistant cells. One member of a family of similar proteins (i.e. abc transporters).

Drugs affected by the MDR phenotype: Doxorubicin, taxanes and vinca alkaloids.

Pre-clinical data: The overexpression of MDR1 protein is associated with drug resistance to relevant substrates; inhibitors of MDR1 can reverse drug resistance in vitro. The roles of the other abc transporters [MRP1 (multiple drug resistance protein 1) and LRP (low-density lipoprotein-related protein 1)] are less clear.

Clinical data: The clinical significance of MDR1 overexpression has been controversial. Good evidence for a significant role exists for acute myeloid leukaemia and myeloma, but the role in solid tumours remains unclear. A recent meta-analysis has demonstrated a role in breast cancer (Ref. 25). Clinical testing of the role of MDR1 by attempts to inhibit MDR1 function have initially been limited by the toxicity of inhibitors, and also by pharmacokinetic interactions that complicate their use. More-potent and less-toxic compounds and drugs, with lesser pharmacokinetic interactions, will allow testing of the clinical importance of MDR1 overexpression.

Conclusion: It is likely that MDR1 has a role in drug resistance in specific tumour types. The effective inhibition of MDR1 in vivo has yet to be demonstrated.

relatively neglected but are probably important. The integration of information from each of these determinants remains problematical.

The importance of a marker of a particular drug-resistance mechanism will depend on the effect that the marker has on chemosensitivity compared with the effects of other competing causes of drug resistance. Ultimately, a more integrated approach is required, one that takes into account: (1) variability in plasma pharmacokinetics, (2) distribution of the drug to the tumour microenvironment, (3) intracellular concentration of the drug and (4) intracellular determinants of drug response.

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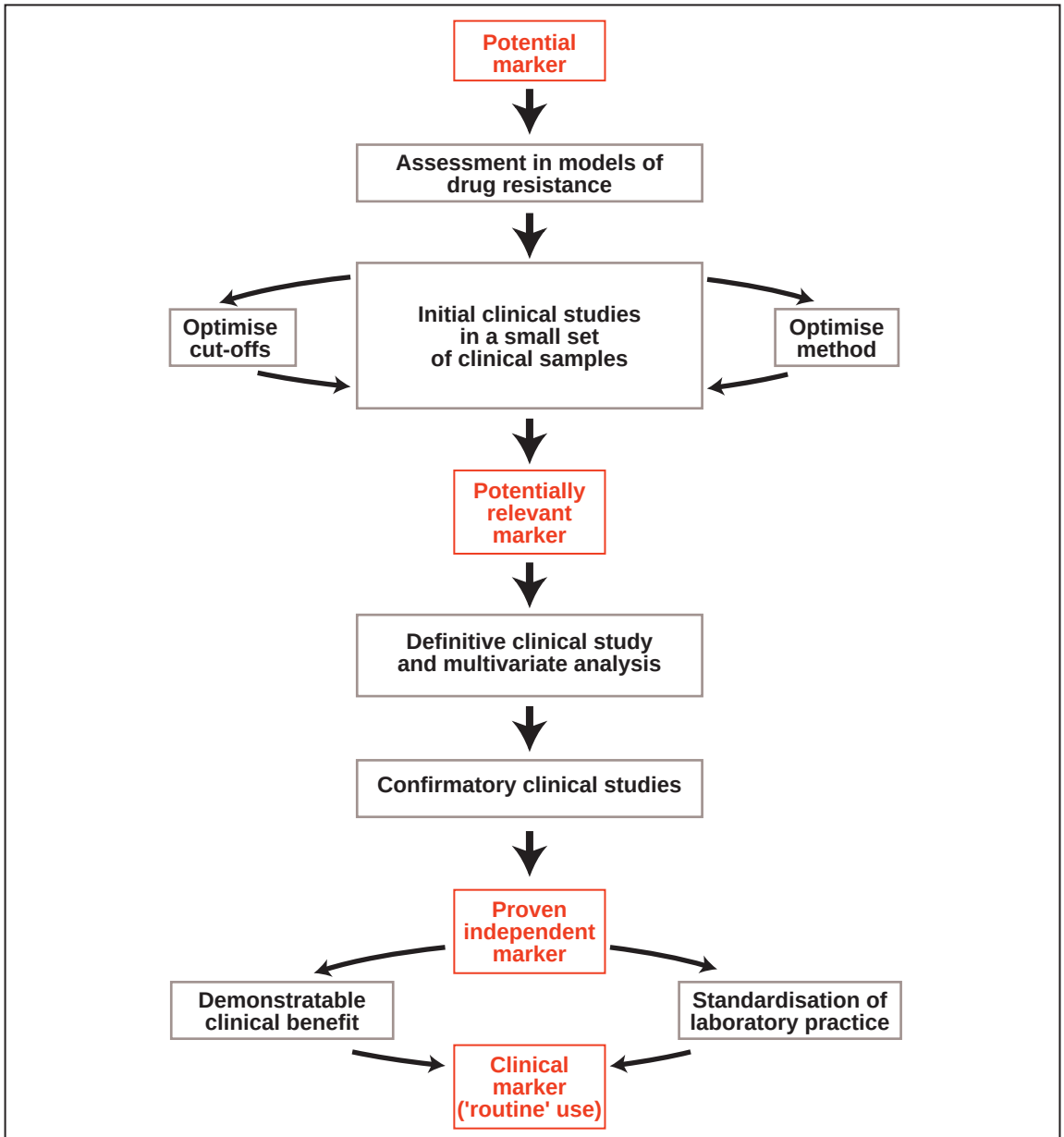

Figure 2. Stages in the development of clinically useful markers of resistance to anti-cancer drugs (*see next page for legend*) **(fig002rbg).**

Evaluation of potential predictors of response

The first question that arises in clinical trials that evaluate potential markers of drug resistance is: 'what is the appropriate clinical or biological endpoint?' A tumour response (i.e. a reduction in the dimensions of a tumour) to a drug is obviously related to drug resistance, but sometimes the analysis of patient survival (or 'disease-free survival') is a more appropriate endpoint. Tumour response can be difficult to measure accurately for some tumours, and those patients who are most likely to benefit from chemotherapy (i.e. those with microscopic disease) cannot

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Figure 2. Stages in the development of clinically useful markers of resistance to anti-cancer drugs. The process has been simplified and presented as a simple, linear progression in the figure; in reality, substantial interactions occur between individual stages. (a) Identification of a potential marker. This occurs through understanding of the basic mechanisms of drug response, serendipity or systematic genome-wide screening. (b) Testing of a potential marker in vitro for a role in drug response. Models, such as matched pairs of parental and resistant tumour-derived cell lines, can be used to determine whether there is an association between expression of the marker and resistance in vitro. Confirmation of a direct role for a marker in drug response can be determined from the effect of biochemical or genetic manipulation of the marker on cytotoxicity. (c) Preliminary studies on clinical samples. The aim of these studies is to: (1) determine that the methodology that is to be used is both practical and reproducible for clinical samples, (2) determine what the optimal cut-offs are for comparison in prognostic studies and (3) identify markers that are of sufficient interest for further investigation in a definitive study. Preliminary studies involve either the comparison of the expression of the marker(s) in sensitive and resistant (either intrinsic or acquired) tumours, or the correlation of the marker with clinical response or disease-free survival after treatment. (d) 'Definitive' studies. Adequately (statistically) powered clinical studies that correlate a marker with clinical drug resistance are required. A multivariate analysis determines whether the measurement of a particular marker provides additional information relative to that provided by other clinical or molecular markers, and generates a model that allows information from multiple markers to be combined. (e) Confirmatory studies. The significance of individual markers, and the validity of derived models, must be confirmed in independent studies using the same procedures and definitions that were used by previous investigators. This determines whether a marker is truly associated with clinical drug resistance. (f) Standardisation of methodology. The implementation of a test method into widespread clinical practice requires the development of a consensus as to the appropriate method for measuring the marker and how to interpret the results obtained. An important issue at this stage is the minimisation of inter-laboratory error. (g) Demonstration of clinical benefit. This can be determined by prospective trials, in which the expression of a marker determines the choice of therapy, or the use of a modulator of drug resistance improves the outcome in selected patients. This last stage overcomes the final barrier to the routine clinical use of the marker **(fig002rbg).**

be directly assessed because their tumours are not measurable using conventional methods. Survival and disease-free survival are well-defined clinical endpoints; however, both are clearly affected by factors that are unrelated to drug resistance but influence other aspects of tumour biology, such as growth or metastasis.

An attempt to clarify the relationship of a molecular marker to drug resistance is demonstrated by the distinction between prognostic factors and predictive factors. A prognostic factor is any variable that provides information about outcome. Prognostic factors can reflect tumour burden or the capacity of a tumour for invasion and metastasis (e.g. vascular invasion); for a 'pure' prognostic factor, this relationship holds irrespective of which type of treatment a patient receives. A predictive factor is a type of prognostic factor that provides information about the outcome for a specific treatment. Such information can help decide which treatment is most effective. Thus, overexpression of the gene encoding the protein MDR1 (Table 2) might be a predictive factor for the survival of patients with ovarian cancer who have been treated with paclitaxel (which is a substrate of MDR1); it would not be a predictive factor for patients who have been treated with carboplatin because carboplatin

Table 3. Specific drug-resistance mechanisms (tab003rbg)

Mechanism	Refs
(1) Drug delivery	
Drug distribution in plasma	34
Drug distribution in tumour microenvironment	35
Drug uptake and efflux	36
(2) Modification of drug effect	
Intracellular inactivation	37
DNA repair	23
Target amplification or modification	38
Altered intracellular signalling	14
Defective p53 signalling	7, 13, 39
Defective mismatch-repair-dependent signalling	40
ERBB2 protein effects	31
Inhibition of apoptosis: BCL2 family	11, 13, 41

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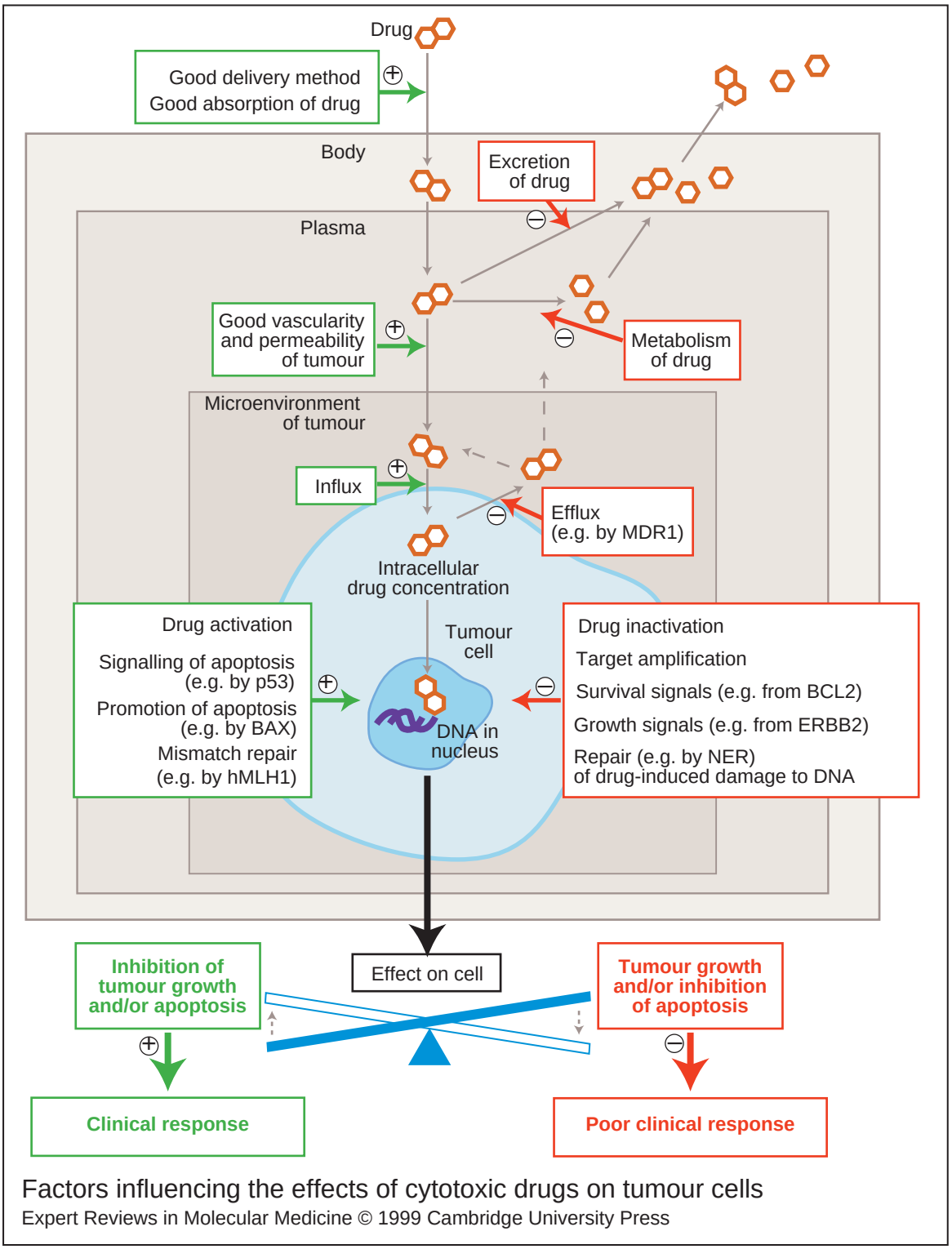

Figure 3. Factors influencing the effects of cytotoxic drugs on tumour cells (*see next page for legend*) **(fig003rbg).**

is not a substrate of MDR1. In practice, the distinction between prognostic and predictive factors is not straightforward, and many factors are a mixture of the two. The best-known example

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Figure 3. Factors influencing the effects of cytotoxic drugs on tumour cells. Multiple barriers exist between the administration of a cytotoxic drug and its final effect. These barriers can occur: (1) before reaching the tumour, and include differences in drug pharmacokinetics in the plasma (drug distribution, metabolism and excretion) or drug distribution to the tumour microenvironment; (2) between reaching the tumour and the target, and include differences in intracellular drug concentration, and drug activation or inactivation and (3) after reaching the drug target, and include alterations in intracellular drug signalling, leading to apoptosis or cell-cycle arrest. At the tissue level, the final effect of the drug is determined by the balance between cell death (by apoptosis or by necrosis) and cell growth. In tumour tissue, the promotion of apoptosis and inhibition of growth is associated with a clinical response (reduction in tumour). The inhibition of apoptosis and promotion of growth are associated with clinical drug resistance. In normal tissues, the promotion of apoptosis and inhibition of growth are associated with the toxicity of normal tissue. Thus, effective cytotoxic therapy relies on the differences in drug delivery and drug response in tumour tissue and in normal cells. Factors that promote cell death are listed on the left of the figure (in green boxes); drug resistance is associated with defects in these pathways. Factors that inhibit cell death or promote cell growth are listed on the right of the figure (in red boxes); drug resistance arises from increased activity of these pathways. Abbreviations used: BAX (anti-apoptotic BCL2 homologue); BCL2 (transforming protein bcl2); ERBB2 [protein tyrosine kinase erb B2 precursor (also known as c-erbB-2 protein precursor or kinase-related transforming protein erbB2)]; MDR1 (multi-drug resistance 1 protein); MLH1 [mutL (*Escherichia coli*) homologue 1]; NER (nucleotide-excision repair); p53 (tumour protein p53) **(fig003rbg).**

is hormone-receptor expression for patients with breast cancer; it is a good prognostic factor for patients with breast cancer who do not undergo hormonal treatment, but also represents a predictive factor for their response to hormone therapy. Many prognostic studies are performed on patients who have received chemotherapy; however, it might be impossible to tell whether a given variable is acting as a prognostic or predictive factor if there are no data for untreated patients or patients who have been treated with drugs that are not affected by the resistance mechanism being studied. In practice, the appropriate clinical endpoint for a predictive marker is 'beneficial response' and disease-free or overall survival of patients. Molecular markers of drug resistance that are prognostic for survival might be predictive factors, but the possibility that they have an effect on prognosis, independent of treatment, must be considered.

Whether a new molecular marker of drug resistance provides additional information that predicts outcome can be evaluated by performing a multivariate analysis to determine whether the marker is an independent predictor, after accounting for existing information. Thus, many of the issues that relate to the evaluation of predictive markers relate to the (statistical) design and interpretation of such multivariate analyses (Refs 4, 5). The final 'proof of principle' of the usefulness of a potential marker comes from a demonstration that the application of the information obtained improves outcome objectively in a prospective trial in which the choice of treatment is determined by expression of the marker.

Problems associated with the evaluation of the clinical significance of a molecular mechanism of drug resistance

Biological, methodological and treatment-related variability

The many potential sources of variability are important in the assessment of predictive molecular markers. This variability can result from both the biology of the marker and the methodology used for its assessment (Table 4). A good example is the assessment of mutation in the human *p53* gene, which encodes the human tumour-suppressor protein p53 (Table 5). p53 is a transcription factor that plays a key role in co-ordinating the arrest of the cell cycle, DNA repair and programmed cell death (i.e. apoptosis) following DNA damage. Many different methods have been used to assess the *p53* gene, including mutation screening, direct sequencing and detection of increased protein expression from the mutated *p53* gene. Although direct sequencing of *p53* is time-consuming and costly, it is the most accurate of the other (more rapid) methods that can be used to screen for *p53* mutations. However, the detection of a mutation in the *p53* gene might not be sufficient to predict response; for example, some researchers have suggested that drug resistance is conferred by mutations at only specific sites within the gene (Ref. 6), and also that defects upstream or downstream of the *p53* gene can result in protein inactivation without *p53* mutation (Ref. 7).

Because of the difficulties associated with the direct detection of *p53* mutations, levels of p53 protein are often measured instead. Increased levels of p53 protein are most commonly the

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Table 4. Differences in the methods used to measure molecular markers of drug resistance (tab004rbg)

Different samples

Protein versus RNA versus DNA: Protein levels do not always correlate with RNA levels or gene amplification.

Different measurement methods

Western blotting versus immunohistochemistry: Western blotting can measure the quantity of protein in homogenised tissue samples, whereas immunohistochemistry can also distinguish between tumour and normal tissue (as well as reveal heterogeneity within tumours).

Different scoring systems

Immunohistochemistry scores can be based on either the intensity or proportion of staining, or a combination of both: This variability in scoring system makes it difficult to compare studies, and multiple comparisons can lead to false-positive results.

Different cut-offs for positive expression: Positive expression can be scored with 'any staining' or 'more than weak staining', which leads to conflicting results.

Membrane expression versus nuclear expression: Positive expression can be scored for either location of protein expression; this leads to difficulty comparing studies, and multiple comparisons can lead to false-positive results.

Different study populations

Type of treatment: Different treatments can be affected by different drug-resistance mechanisms.

Stage: Late-stage tumours can accumulate additional genetic defects, which might not be present in early-stage tumours.

Table 5. p53-dependent cell signalling and drug resistance (tab005rbg)

p53 protein: p53 (tumour protein p53) is a human tumour-suppressor protein. It acts as a transcription factor, playing a key role in co-ordinating the arrest of the cell cycle, DNA repair and programmed cell death (i.e. apoptosis) following DNA damage. More specifically, the activation, phosphorylation and stabilisation of p53 increases the transcription of the regulatory targets that are involved in cell-cycle arrest (e.g. p21), apoptosis (e.g. BAX) and DNA repair (Refs 7, 39).

Relevant drugs: p53 is activated by most cytotoxic drugs, DNA damage, microtubule damage and hypoxia.

Pre-clinical data: The inactivation of p53 is associated with resistance to DNA-damaging agents, although increased sensitivity to these agents can be demonstrated under some experimental conditions. Cell lines that have mutant p53 are generally more resistant to DNA-damaging drugs; however, the overlap with the sensitivity of cells that have wild-type p53 is wide.

Clinical data: There have been reports that the overexpression of p53 protein is significantly associated with drug resistance, or drug sensitivity or that it is not associated with clinical drug resistance. There is some evidence that *p53* mutational status or even mutations in specific regions of the *p53* gene correlate better with drug resistance. Most studies do, however, favour an association between the overexpression of p53 and drug resistance.

Conclusion: Results of studies of p53 and/or *p53* mutations are extremely dependent on their molecular context; clearer relationships might emerge with the improved definition of clinical groups.

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result of a gene mutation prolonging the half-life of the protein; however, the correlation between elevated levels of p53 protein and *p53* gene mutation is not perfect (Ref. 8). Protein expression in a cell or cell extract can be measured by determining the degree of binding of specific antibodies to the protein, using either western blotting or immunohistochemistry. If immunohistochemistry is used, the presence of p53 in the nucleus, or throughout the cell, can be assessed. Various definitions of positive immunostaining have been used; these depend on the intensity of staining, the percentage of cells stained, or various indices that are derived from the intensity and proportion of staining. For example, weak staining may be considered either positive or negative. None of these methods examines directly the relationship between p53 protein levels and actual function of the protein. Similarly, DNA amplification, total protein levels and specific membrane immunoreactivity have all been used to assess the expression of the *ERBB2* gene. It is not surprising, therefore, that conflicting results have been reported.

Another source of the conflicting results arising from studies of molecular markers in drug resistance is the between-study variability in crucial biological factors. Different study populations vary with regard to clinical prognostic factors (e.g. stage of cancer) and biological factors (e.g. prior exposure of the patient to carcinogens). *p53* mutations tend to correlate with stage for many tumours, and advanced-stage tumours with *p53* mutations might have accumulated additional mutations in other genes, owing to the loss of the p53-regulated mechanism that repairs DNA damage. Thus, studies of the prognostic significance of *p53* mutations can differ between patients with early-stage tumours and those with metastatic disease.

There are many reasons why the spectrum of mutations seen in a tumour can differ between study populations; for example, the age, environmental exposures and genetic backgrounds of the populations can differ. Differences in input from molecular pathways, which are not being taken into account, can modify the significance of the molecular mechanism that is being studied. Thus, results can be generally applicable only if they have been reproduced in more than one patient population.

The heterogeneity of marker expression within a tumour is a complex cause of variability. This is because the sub-population of tumour cells that is resistant and actually determines outcome can be quite small and difficult to detect. An example of variability within a tumour was recently demonstrated in one study that showed differences in the expression of thymidylate synthetase (a potential predictor of response to the cytotoxic drug 5-fluorouracil) between primary colorectal cancers and their metastases (Ref. 9). This study highlighted the fact that the expression of markers within a tumour cannot be assumed to be constant, and also that the measurement of the expression of markers in a primary tumour might not predict the response at other tumour sites.

An extremely important cause of variability between studies is that due to differences in the treatments used between and within studies. Studies of archived tumour specimens often include specimens from patients who have received a wide variety of treatments or those whose treatment regimens are unknown. However, predictive factors that are relevant for one type of treatment might not be significant for another type of treatment that is being used for the same type of cancer. An example of this is ovarian cancer; combinations of taxane and platinum are now replacing previous 'standard' treatment regimens that consisted of platinum in combination with alkylating agents. Resistance mechanisms for taxanes, such as MDR1 (Table 2) and alterations in β-tubulin (Ref. 10), are different from those for cisplatin and alkylating agents, such as p53 (Table 5) and mismatch repair (Table 6). Clearly, the evaluation of the prognostic significance of any of these factors requires the standardisation of the treatment received.

Complexity and redundancy

Many of the developments in the understanding of drug-resistance mechanisms have highlighted some major difficulties. The first difficulty is the enormous complexity of individual systems; a second problem is the redundancy demonstrated by many systems. The regulation of apoptosis by the family of human *BCL2* genes (Table 7) represents a good example of both of these problems (Ref. 11). The identification of the BCL2 protein, which can cause drug resistance in vivo and is an inhibitor of apoptosis, provided an entirely new drug-resistance mechanism (Ref. 12).

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Table 6. Mismatch repair and drug resistance (tab006rbg)

Mismatch repair: The mismatch-repair function is mediated by a family of genes; these genes are expressed in a wide range of tissues, and are involved in the repair of common, single base-pair mismatches that can occur during DNA replication. If a functional mutation occurs in one or more of these genes, the errors in DNA replication can accumulate and eventually contribute to transformation of the cell. Failure of mismatch repair is thought to be a common mechanism in inherited and sporadic cancer development, and was first identified in inherited colo-rectal cancer.

Role of mismatch repair in drug resistance: Mismatch repair proteins are able to recognise and bind to DNA damage. Binding to these proteins is thought to elicit either futile cycles of mismatch repair or replication stalling, which can lead to cell death. Thus, in the absence of the recognition of DNA damage or the engagement of cell death, cells become resistant (or tolerant) to the DNA damage.

Relevant drugs: Cisplatin, mono-functional alkylating agents and doxorubicin (Refs 24, 40).

Pre-clinical data: Some drug-resistant cell lines are associated with the inactivation of the mismatch-repair function; reversal of this defect can partially reverse drug resistance.

Clinical data: Preliminary evidence shows that a loss of MLH1 protein expression (possibly due to methylation of the *MLH1* gene promoter) correlates with a poor outcome in patients with ovarian (Ref. 42) and breast cancer (Ref. 22) who are treated with DNA-damaging drugs.

Conclusion: There is a probable role for mismatch repair in mediating some clinical cisplatin resistance; however, its prognostic significance needs to be confirmed in large multivariate analyses.

Table 7. BAX and BCL2 family of proteins and drug resistance (tab007rbg)

***BCL2*:** *BCL2* (also known as B-cell CLL/lymphoma 2; *bcl-2* and *BCL-2*) is a proto-oncogene that encodes BCL2 (transforming protein bcl2), a 25-kDa protein that inhibits apoptosis (Refs 11, 12, 41).

Role of BCL2 in drug resistance: BCL2 is part of a large family of related proteins that either inhibit (e.g. BCL2 and BCL-Xs) or promote (e.g. BCL-xl and BAD) apoptosis. It has been proposed that BCL2 and BAX (BCL2-associated X protein) form heterodimers, and that the activity of the apoptosis system is determined by the balance between the two components.

Pre-clinical data: There are conflicting data on the correlation between the expression of BCL2 protein family members in drug-resistance models; however, transfection experiments have demonstrated roles for some family members in the modulation of drug-induced apoptosis.

Clinical data: There are contradictory results with BCL2 protein expression and its association with improved outcome; this contradiction might be due to an association with an increased rate of cell proliferation (Ref. 15). There are some preliminary clinical data on the roles of other members of the BCL2 family of proteins.

Conclusion: There is a potential role for BCL2 family members in drug resistance but further work is required to determine which ones actually determine activity in specific tumours and with which drugs.

However, subsequent investigations have shown that the regulation of apoptosis by this system does not depend on BCL2 protein alone. The predisposition to apoptosis is determined by the balance between anti-apoptotic proteins (e.g. BCL2, BCL-Xl and BCL-w) and pro-apoptotic proteins (e.g. BAX, BCL-Xs, BAK and BAD). Measuring a single component gives only limited information on the entire system, unless there is evidence that variation in that component determines overall system function. Clearly, there is a strong selective pressure for tumours to evolve multiple mechanisms for escaping apoptosis, and there needs to be a strong biological rationale for choosing which part(s) of the apoptosis system to measure.

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Another example is the p53 pathway (Table 5): p53-dependent signalling is an important determinant of apoptosis after chemotherapy (Refs 13, 14). However, it has become increasingly clear that partly redundant p53-independent mechanisms of cell death can occur, which might limit the significance of the inactivation of p53 protein. In addition, most of the published studies have considered the inactivation of p53 to be the result of mutation, whereas it is now known that the p53 system can be inactivated by other methods; these include attenuated upstream activation [e.g. involving the ataxia telangiectasia mutated protein (ATM protein)] and downstream inactivation of p53 by, for example, the human homologue of mouse double minute 2 (MDM2; Ref. 8). The redundancy and complexity of drug-response systems must be considered in any assessment of the prognostic significance of individual markers.

Interdependence of systems

One major difficulty of assessing the importance of individual predictive molecular markers is the interdependence of the biological systems that are involved. For example, an intact p53 pathway is an important determinant of apoptosis; however, whether apoptosis will follow the induction of p53 depends on the balance between BAX and BCL2 (Ref. 10). In addition, *p53* mutations lead to downregulation of the expression of the *BAX* (BCL2-associated X protein) gene; thus, it is difficult to interpret the prognostic significance of BAX protein levels without knowing whether this is a secondary effect of *p53* mutation.

Similarly, there is an interaction between the regulation of apoptosis by BCL2 protein (Table 7) and the regulation of cell proliferation (Ref. 15). The expression of the *BCL2* gene is associated with a low-proliferative (S-phase) fraction. This might explain the unexpected results of some studies of breast cancer, where overexpression of the *BCL2* gene was associated with a good prognosis (due to slow tumour growth) instead of a worse prognosis (due to drug resistance). These examples highlight the fact that the interdependence of pathways that are involved in drug resistance means that changes in predictive markers cannot be considered in isolation. This again emphasises the need to measure multiple pathways and to understand the interactions between them.

Dealing with multifactorial determinants of drug responses

Given the multiple, complex and interdependent determinants of the drug response by tumour cells, it seems reasonable to assume that clinical drug resistance is multifactorial. The identification of multiple predictive molecular markers complicates the integration of results: for example, what does it mean if a particular tumour has five 'good' prognostic factors and five 'bad' ones? It is necessary to collate the information from multiple predictive factors, both clinical and molecular. This requires knowledge of the significance of different pieces of information and also how much statistical 'weight' should be assigned to each piece. Such weights can be determined from models that are developed using multivariate analysis; however, such a model needs to be validated on an independent data set.

One under-emphasised issue that is associated with the interpretation of multiple significant factors is the consideration of how much of the variation in drug response or patient survival is explained by the model. The prognostic models that have been used so far explain only 10–50% of the variation that has been observed for patient survival, despite the identification of multiple and significant clinical prognostic factors (Ref. 16). This disappointing conclusion is because many markers apply to only a minority of patients (Ref. 17). This emphasises the fact that the identification of significant prognostic markers in a multivariate analysis does not necessarily mean that the model usefully predicts response.

Statistical issues: multiple testing, overfitting and underpowering

Several issues that relate to the design of clinical trials and the statistical analysis of their results contribute to the conflicting results arising from prognostic studies (Refs 4, 5); these include multiple testing, overfitting and underpowering.

A particularly important issue is that the design of many prognostic studies can lead to multiple comparisons being made; for example, multiple potential clinical markers can be tested and results can be expressed in multiple ways, which means that a large number of comparisons are made. The 'overfitting of the data' is a problem that results from the inclusion of multiple potential factors in a small data set, and increases the probability that a marker

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is assessed to be significant on subsequent multivariate analysis. Multiple comparisons and overfitting increase the probability of a false-positive result. There is an important distinction between preliminary and confirmatory studies, as defined by McGuire (Ref. 1), with respect to the validity of multiple comparisons. A hypothesis-generating preliminary study might make multiple comparisons to identify candidate markers of drug response; however, no definitive judgements about the validity of such markers can be made. A confirmatory study needs to focus on the validation of a specific marker, and should not involve multiple comparisons.

Underpowering refers to the performance of small studies that do not have the statistical power to detect the association of interest, and results in false-negative results. A combination of an adequately powered 'positive' study and numerous underpowered 'negative' studies leads to confusion in the literature and valid observations being discounted.

Clinical trials of modulators of drug resistance

The improvement of prognosis by the reversal of a particular drug-resistance mechanism in the clinic is the ultimate proof of principle of the importance of that mechanism. Several difficulties are encountered when conducting such clinical trials; they are similar to those encountered during the evaluation of other non-cytotoxic compounds (e.g. angiogenesis inhibitors), and have been widely discussed recently (Ref. 18). The major difficulty is that the endpoint of a trial of a cytotoxic–modulator combination is an improvement in its anti-cancer cytotoxicity compared with that of the cytotoxic drug on its own. This increased effectiveness of the combination should be specific for the tumour, relative to its toxicity in normal tissues. Comparisons of the effects of different types of treatment are problematic, particularly in preliminary 'dose-finding studies', because they require large numbers of patients to be studied. The development of resistance modulators is greatly aided by the development of validated 'intermediate endpoints' that correlate with the desired biological effect (e.g. inhibition of an enzyme or attainment of a specific plasma concentration). Thus, dose-finding studies using a small cohort can be used to determine the intermediate endpoint, before evaluating the efficacy of the modulator in a large randomised clinical trial.

A second issue is the decision about which patients should be included in efficacy trials. Preliminary work needs to determine the role of the resistance mechanism in a variety of tumour types and stages, so that modulators can be tested on an optimal target population.

Solutions to the problems associated with the evaluation of the clinical significance of a molecular mechanism of drug resistance

Standardisation and validation of results from studies using the same methods and treatment populations

The two most important requirements for a prognostic marker of drug resistance are: (1) that the methodology that is used to measure it is reproducible between laboratories and (2) that any preliminary positive report is confirmed in a different patient population. The comparison of results from different studies is limited by the use of different methodologies, in particular the use of different 'cut-offs' for variables. For example, in ovarian cancer cell lines, the loss of expression of the mismatch-repair protein, MLH1, correlates with resistance to cisplatin (Table 6). One scale that has been used to measure and assess the degree of immunostaining of MLH1 protein allocates samples a score between 0 and 6; different cut-off points between high and low expression can be used when comparing groups (e.g. 0, –1 versus 2–6 or 0–3 versus 3–6), and this choice is critical. It is not valid to test multiple cut-offs and choose the one with a positive result; often the median value is used, but this lacks biological significance. Sophisticated statistical methods can be used to aid the choice of an appropriate cut-off. An alternative approach involves the validation of the chosen cut-off by correlating it with actual function. Thus, the assignment of immunostaining scores for the expression of MLH1 protein so that they correlate with loss of mismatch-repair function will result in a more logical choice of an appropriate cut-off. Regardless of the chosen cut-off point, it is extremely important that consistent definitions and methods are used in subsequent confirmatory studies.

The minimisation of patient variability and treatment variability is also facilitated by studying populations of patients who have well-defined

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clinical characteristics and who have been rapidly recruited to prospective clinical trials and treated with standardised treatment regimens.

Choice of 'ideal' patient populations and populations with reduced variability

A major problem that is faced during the evaluation of predictive markers of drug resistance is the biological variability that exists between patients; however, several recent reports have suggested how this problem might be minimised. One approach is to select a homogeneous group of patients on the basis of clinical or biological criteria. The elderly represent a clinically identifiable group for which a high incidence of drug resistance is associated with many different cancers. In one study, the overexpression of MDR1 protein (Table 2) was shown to correlate with a high rate of treatment failure for elderly patients with acute myeloid leukaemia (Ref. 19). An example of a biologically selected group is the selection of a group of patients with tumours that overexpress the ERBB2 protein (Table 1) for treatment with an antibody against ERBB2. The identification of 'ideal' populations such as this one, especially those that are characterised by even more homogeneous resistance mechanisms, is particularly important when planning clinical trials of resistance modulators.

A second strategy uses a two-step approach: first, an intervention is used to overcome or reverse a major resistance mechanism in a population, before studying this population (which has reduced variability) to identify other resistance mechanisms. For example, pharmacokinetic variability (i.e. differences in elimination) for carboplatin (an analogue of cisplatin) can be markedly reduced if drug doses are adjusted according to rate of excretion of the drug from the kidneys, which correlates with kidney function. Similar approaches to the minimisation of pharmacokinetic variability are becoming available for other drugs.

Clinical trials of agents that have been designed to reverse drug resistance result from the overexpression of either ERBB2 (Refs 20, 21) or MDR1 can also produce a population for which one of the major sources of variability in treatment response has been minimised. Such ideal populations are suitable for exploring the significance of unrelated drug-resistance mechanisms.

Accounting for within-tumour heterogeneity

Variability in the expression of resistance markers within a tumour (intra-tumoral heterogeneity) is an extremely difficult problem because the cells that determine resistance might represent a small sub-population of cells that are difficult to identify among the more frequently occurring sensitive tumour cells; furthermore, variability can occur between different tumour sites. However, several approaches have been proposed in an attempt to deal with this issue. Obviously, it would be preferable to measure resistance-marker expression at the site of interest; however, this is not always feasible, especially if there are multiple metastatic sites. Methods such as immunohistochemistry and flow cytometry, which allow the measurement of resistance-marker expression in populations of cells, can be used to help identify sub-populations of cells that have variant expression. If immunohistochemistry is used, it is important to determine whether it is the average level of expression of a marker or the proportion of cells with a high or low level of expression of the marker that predicts the response.

An innovative means of addressing the problem of intra-tumoral heterogeneity is to use the technique of in vivo clonal selection of resistant cells. The biopsy of residual tumour that remains after chemotherapy cannot be used to **predict** drug resistance; the presence of a tumour mass after chemotherapy **defines** clinical drug resistance. However, biopsies of residual tumour masses that remain after chemotherapy can help to determine the mechanism of drug resistance and thus the choice of subsequent treatment. A tumour mass that is present after chemotherapy will contain a cell population that has been 'enriched' for cells that express drug-resistance markers. In a study that examined the prognostic significance of the expression of MLH1 protein for future response to DNA-damaging chemotherapy in breast cancer, the level of MLH1 protein expression in the residual tumour predicted a poor survival; by contrast, the level of MLH1 protein expression in the initial tumour biopsy did not (Ref. 22).

Sampling each independent system involved in determining response

The response of cancer cells to anti-cancer drugs can be modelled as a series of interdependent

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Table 8. Nucleotide-excision repair and drug resistance (tab008rbg)

Nucleotide-excision repair: A multiprotein complex that is involved in the recognition of DNA damage, excision of the damaged strand, and synthesis and ligation of a new strand. ERCC1 is a key protein in this complex; other proteins involved in the complex include XPA.

Role of nucleotide-excision repair in drug resistance: Although evidence of loss of nucleotide-excision repair has been demonstrated in cells that are hypersensitive to DNA-damaging agents such as cisplatin, its role in drug resistance remains controversial. Evidence of released ERCC1 correlating with resistance to cisplatin has been observed.

Relevant drugs: Platinum adducts (Ref. 23).

Pre-clinical data: The reduced removal of platinum adducts has been seen in some resistant cell lines. Contradictory results have been obtained for the role of nucleotide-excision repair in drug resistance, using direct assays of nucleotide-excision repair activity in resistant cell lines. ERCC1 appears to be a good surrogate marker of nucleotide-excision repair activity.

Clinical data: In some small studies, the overexpression of ERCC1 correlated with resistance to platinum-based therapies.

Conclusion: There is a probable role for nucleotide-excision repair in mediating some clinical resistance to cisplatin; however, large multivariate analyses are required to confirm its prognostic significance.

systems (or molecular pathways) that have multiple components. The selection of components for evaluation as predictive factors is important, and has usually been based on empirical and pragmatic grounds. The development of models that explain the interactions that occur between pathway components (and between pathways) makes a model-based approach feasible. The development of a model requires three steps: (1) the determination of which pathways (or systems) are important for mediating drug resistance, (2) the determination of which components are the key determinants of system function and (3) the integration of the information from each key component into a prognostic model. For example, the following pathways have all been implicated in determining the response of cancer cells to cisplatin: intracellular drug inactivation, p53-dependent signalling (Table 5), mismatch repair (Table 6), nucleotide-excision repair (Table 8) and interactions of the BCL2 family of proteins (Table 7). Thus, an ideal prognostic study would systematically analyse the most important determinants of each of these systems.

Choice of molecular markers that determine system (pathway) function

The choice of which molecular marker to measure when faced with complex, interdependent and overlapping molecular systems is helped by having an understanding of which of the variables determines the function of each system (pathway). The role of the ERCC1 [excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)] protein in nucleotide-excision repair is a good example (Table 8; Ref. 23). Nucleotide-excision repair is a complex system that repairs DNA damage caused by cisplatin; however, evidence is accumulating to suggest that the measurement of the level of ERCC1 protein alone can predict the response of cells to cisplatin, and correlates well with nucleotide-excision repair activity. These observations might be due to the co-ordinated regulation of the transcription of related proteins; thus, the measurement of the expression of one such protein is a good surrogate for the expression of the others. Alternatively, the probability of an inactivating event occurring during nucleotide-excision repair might be greatest for a particular component of a pathway. For instance, in the case of mismatch repair (Table 6), reduced levels of MLH1 protein, rather than other components of the pathway, were frequently observed in cells that were selected for resistance to drugs such as cisplatin (Ref. 24). Thus, a good understanding of which variables determine pathway function means that the

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analysis of a few carefully selected markers can be used to summarise the effects of complex systems.

An interesting approach to the simplification of the study of complex systems is to measure downstream events that summarise the input or pathway system. Examples of this approach include the measurement of: (1) p21 protein levels, (2) DNA adduct levels and (3) cell proliferation. The measurement of p21 protein levels can provide an in vivo (downstream) summary of p53 function; however, its prognostic significance is complicated by cross-talk from p53-independent pathways.

The cytotoxic lesion following treatment with cisplatin is a covalent bond between cisplatin and DNA (DNA adduct). The measurement of DNA adduct levels in leucocytes (white blood cells) after treatment with cisplatin can, in some tumours, be a predictive marker for the response of tumour cells to cisplatin. Thus, DNA adduct levels provide information about drug uptake into tumour cells, drug inactivation and adduct removal by DNA repair and apoptosis, rather than a direct measure of the activity of a single pathway (Ref. 23). The measurement of cell proliferation by calculating the S-phase fraction can represent a summary of the information about the activity of multiple oncogenes and cell-cycle pathways in the cells. Unfortunately, the measurement of cell proliferation has tended to be unreliable.

The development of such summary indicators is greatly aided by the development of functional assays that can be used with clinically available tissue, including biopsy tissues. In some ways, pathological markers, such as tumour grade and histological type, already act as summary markers of the input from multiple molecular systems. The challenge is to understand these pathways and their interactions, and to use this understanding to choose molecular markers that summarise input from multiple relevant systems.

The use of co-operative groups for adequately powered prognostic studies

A major limitation of many of the studies reported in the literature has been the problem of small (i.e. statistically underpowered) studies giving false-negative results. For example, the confirmation of a predictive role for ERBB2 protein to predict the response to anthracycline-based chemotherapy in node-positive breast cancer has required multiple, well-designed trials involving thousands of patients. This has been achieved as a result of the efforts of collaborative groups such as the Cancer and Leukemia Group B (CALBG) and National Surgical Adjuvant Breast and Bowel Project (NSABP; see Table 1) amongst others. To date, co-operative groups have been required to provide sufficient numbers of patients, to answer most clinically important research questions; in the future, such collaboration will undoubtedly be required to answer questions regarding the importance of predictive markers.

The use of meta-analysis to help determine sources of variability and account for conflicting studies

Another method that is a useful adjunct to adequately powered studies is the performance of well-conducted meta-analyses. Meta-analyses are a group of well-defined statistical techniques for combining results from multiple studies. A recent meta-analysis of all of the published studies investigating the role of the MDR1 protein (Table 2) in breast cancer provided strong evidence for its significance, and identified important sources of heterogeneity in previously reported studies (Ref. 25). Meta-analyses would, no doubt, contribute to the understanding of similar problems in those instances where different studies produce conflicting results (e.g. p53 and its relevance to drug resistance). Meta-analysis of these types of studies does, however, present some extremely difficult methodological problems, which relate particularly to combining information from studies with different methodologies and patient populations (Ref. 5).

Categories, formulas and neural networks: construction of prognostic indexes or equations

The ultimate usefulness of information generated by the study of drug-resistance markers for the determination of prognosis in individual patients is limited by our ability to make use of such information. In practice, clinical prognostic factors have mostly been incorporated into: (1) categories, such as the Nottingham Index, a validated index for early breast cancer (Ref. 26); (2) formulas, such as those that are available for germ-cell tumours (Ref. 27) or (3) neural networks, which use sophisticated computer-generated algorithms to predict prognosis (Refs 28, 29). Such indexes must be validated on a different patient

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population to that on which they were derived (i.e. an independent data set). These indexes have become useful tools for predicting prognosis from clinical variables. Prognostic indexes are popular with clinicians because of their simplicity and ease of use in a clinical setting. The successful utilisation of multiple sources of additional prognostic information will undoubtedly require the development of more complex and sophisticated tools, such as prognostic equations or neural networks.

Research in progress

Research now in progress is focused on the clarification of the importance of potential markers that have been identified in pilot studies. Many areas of controversy have not been investigated in adequately sized studies of patients who are receiving similar treatment. This issue is now being addressed by the analysis of samples that have been collected as part of studies being carried out by large co-operative groups. Meta-analysis might be expected to resolve areas of controversy and to identify causes of heterogeneity between different studies. The role of the MDR1 protein as a molecular marker of drug resistance should become clearer on the completion of phase III trials of the effects of new agents (e.g. PSC833) that are able to reverse this mechanism of drug resistance.

Future directions

Genome-wide screening methods

The development of gene-array technology has important implications for the assessment of molecular markers of drug resistance (Ref. 30). The ability to automate the assessment of changes in genome-wide gene expression (i.e. throughout the genome) will not only generate large numbers of novel potential molecular markers but also facilitate their evaluation for clinical significance. However, this technology is still in its infancy, and substantial issues relating to its application to clinical samples, its cost and data management need to be addressed.

Automated methods of examining genetic profiles using microsatellite markers, or of examining gross chromosomal changes using comparative genomic hybridisation, also offer a means of correlating genetic changes in tumours with response to chemotherapy. No doubt such methods will generate many more potential markers. The choice of which markers should be further investigated in confirmatory studies, and the development of practical means of utilising the resultant information, will become increasingly important.

Therapeutic intervention in drug resistance

The ability to reverse specific genetic abnormalities, for example by gene transfer or the administration of specific inhibitors, provides an opportunity to test the significance of individual molecular mechanisms in vivo in a way that has not previously been possible. The development of drugs and small molecules that inhibit specific pathways points to a novel approach for the reversal of drug resistance by modulating drug-induced signalling. In addition, clinical trials in which individual resistance mechanisms have been reversed will provide ideal populations for the assessment of the roles of other resistance mechanisms.

Novel drugs with specific biological targets

Finally, there has been a dramatic change in the types of compounds that are being evaluated in early clinical trials. Novel drugs have been designed against specific biological targets; these targets include: (1) specific oncogenes (e.g. the development of farnesyly transferase inhibitor drugs), (2) signal transduction (e.g. the development of epidermal growth factor receptor antagonists), (3) cell-cycle checkpoints (e.g. the use of flavopiridol or 7-OH-stauropsorine drugs) and (4) mediators of tumour invasion and metastasis (e.g. the use of metalloproteinase inhibitors and angiostatin as a drug). However, novel mechanisms of resistance against these novel drugs will probably evolve. Thus, if these 'rational' drugs, which target specific biochemical pathways, are to be used effectively in cancer treatment, an increased understanding of how the pathways function and interact will, no doubt, be required to avoid or minimise the development of drug resistance.

Summary

The complexity, redundancy and interdependence of biological systems determine how a tumour responds to chemotherapeutic agents, and are major barriers to the research and clinical assessment of molecular markers of drug resistance. The development of practical

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prognostic models is dependent on an understanding of which molecular systems determine the drug response and which measurements determine system function. Such models must summarise input from multiple systems, and require a comprehensive assessment of clinical and molecular prognostic factors. Recent developments in our understanding of basic mechanisms of drug resistance, coupled with improvements in the evaluation of potential markers of drug resistance, suggest that this goal is achievable.

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Further reading, resources and contacts

The Tumor Gene Database provides useful summaries of genes of interest.
http://condor.bcm.tmc.edu/ermb/tgdb/

The home page of the p53 Web site.
http://perso.curie.fr/Thierry.Soussi/

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