Review Series: Bench to Bedside

Biomarker profiling for cancer diagnosis, prognosis and therapeutic management

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ABSTRACT

The basis of cancer treatment has come a long way since the days of the the classical TNM staging. Identification of novel biomarkers for various cancers and their specific correlations with prognosis have increased our understanding of carcinogenesis and tumour progression on the molecular level. Recent advances in technologies for simultaneous detection of multiple biomarkers have opened up new avenues for multimarker profiling on a more individual scale. We summarize the key molecular determinants and their prognostic role in various cancers, and examine the available techniques for analysing biomarker panels that can have a major impact on cancer diagnostics and therapeutics.

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INTRODUCTION

Pathological staging of cancers traditionally takes into account the size of the tumour (T), involvement of lymph nodes (N) and presence of distant metastases (M) in the formulation of the TNM stage. While other specialized systems of classification exist, such as the International Federation of Gynaecology and Obstetrics (FIGO) system for cervical cancers and the Duke's system for colon cancers, their central theme is still based on the concepts of extent of the tumour, involvement of lymph nodes, and local and regional spread. Accurate staging of tumours is also important for determining the prognosis, selection of appropriate therapy and estimation of therapeutic response.

The importance of recognition of concerted morphological and biochemical changes such as angiogenesis and elevation of levels of serum markers during tumour development has been known for decades. The presence of biomarkers has often been incorporated in different staging criteria. Microvessel density is measured as an indicator of active tumour angiogenesis and has been correlated with the incidence of metastases.[1] Serum markers such as prostate-specific antigen (in prostate cancer)[2] alpha-foetoprotein (in primary hepatomas and germ cell tumours),[3] and carcinoembryonic antigen (in colorectal, pancreatic, breast and lung cancers)[4] have often been used in the diagnosis and follow up of patients, with extensive investigations being carried out on their prognostic and therapeutic value. The advent of molecular markers is relatively recent, though investigations into a single biomarker or a small combination of biomarkers for specific cancers have been successful in determining their value in the prediction of prognosis and outcome. 'Patterns of expression' or 'profiles' of multiple such molecular markers at the transcript and protein levels (which are increasingly being analysed quantitatively) are now being seen as potential tools for classifying tumours into more logical tiers based on their risk for progression and expected clinical outcome, thereby paving the way for more rational therapeutics.

KEY MOLECULAR DETERMINANTS OF CANCER PROGRESSION

Cancer has been known to have a multifactorial basis, and as our knowledge of the various molecular events involved increases, so do the number of studies pertaining to their correlation. It is beyond the scope of this review to discuss at length the various molecular markers involved in different cancer subtypes. However, it is interesting to note the specific interactions between certain molecules generally studied in most major tumours. This review will briefly delve into the studies on molecules involved in major pathways controlling cancer progression such as cell cycle regulation, apoptosis (programmed cell death), angiogenesis and metastasis. Key molecules participating in these processes are briefly reviewed below and schematically represented in Fig. 1.

Control of the cell cycle is considered crucial in governing a cell's decision to commit to DNA synthesis and proliferation, versus growth arrest when a cell suffers genomic lesions, leading to either repair of damaged DNA or apoptosis. The various oncogenes and tumour suppressors that control this phase are therefore central targets for genetic alterations in human cancers.[5] The *TP53* tumour suppressor gene is central in the cell cycle and apoptotic pathways, and encodes the p53 protein that inhibits phase-specific cell cycle progression by blocking the G1–S transition.[6] Nuclear localization of the p53 molecule is essential for its activity[7,8] and the wild-type protein has a short half-life of <30 minutes. Hence, nuclear accumulation of the protein, as detected by immunohistochemistry (IHC), has been hypothesized to correlate with loss of function of the protein.[9] The effect of p53 is mediated through the transcriptional activation of its downstream effecter, p21.[10] p21 is a cyclin-dependent kinase (CDK) inhibitor and its reduced expression has been reported to be of prognostic value in several human malignancies. However, the expression of

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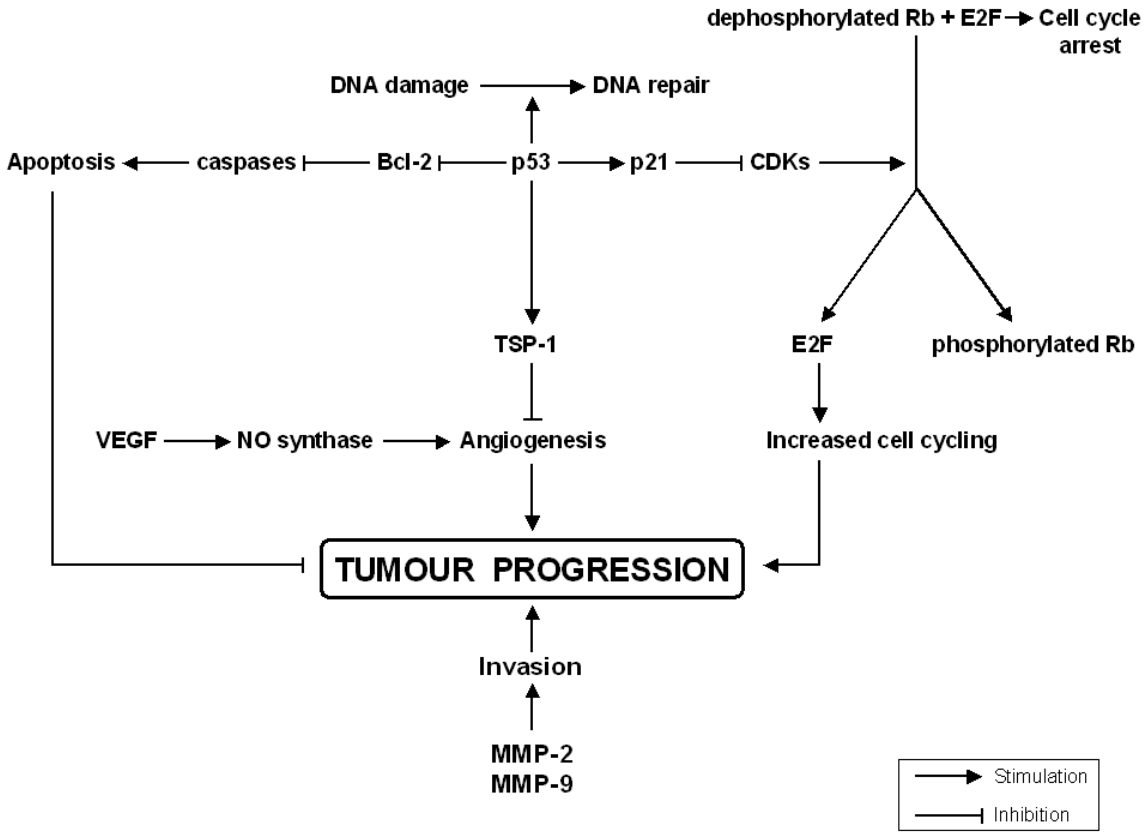

FIG 1. Salient molecular players in cancer progression. Rb retinoblastoma CDK cyclin-dependent kinase TSP-1 thrombospondin-1 VEGF vascular endothelial growth factor MMP matrix metalloproteinase

p21 may be mediated by p53-independent pathways as well, as demonstrated by Li *et al.*[11]

The retinoblastoma gene encodes a nuclear phosphoprotein (pRb), which in the hypophosphorylated form binds and sequesters the transcription factor E2F (Fig. 1).[12] Phosphorylation of pRb by CDKs releases the bound E2F that can mediate the transcription of genes for DNA synthesis.[13] The tumour suppressive role of the pRb protein in controlling the cell cycle through its regulation of E2F is, therefore, extremely crucial. The CDKs that are pivotal for functional regulation of pRb are themselves negatively regulated by CDK inhibitors which include p16, p27 and p21, of which p21 is a downstream effector of p53, thus tying in the two suppressor function pathways.

p53 also regulates apoptosis by induction or repression of downstream effecter molecules such as B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax).[14] Bcl-2 is the prototype for a family of genes that are anti-apoptotic and inhibits the activation of downstream caspases which, when otherwise stimulated, can trigger the apoptotic cascade.[15]

p53 also plays a role in angiogenesis by stimulating thrombospondin-1 (TSP-1) which acts as a potent angiogenesis inhibitor.[16] The action of TSP-1 is countered by the pro-angiogenic effects of the vascular endothelial growth factor (VEGF) that exerts its effects by upregulating nitric oxide (NO) synthase which in turn stimulates NO formation and tumour vascularization.[17] It is the subtle balance between the pro- and anti-angiogenic factors that determines the ultimate vascularity of the tumour.

Special attention also needs to be devoted to the class of matrix metalloproteinases (MMPs), specific proteolytic enzymes that degrade the extracellular matrix and can contribute to increased propensity for tumour invasion.[18] The following section will focus on the studies specifically conducted on MMP-2 and MMP-9 in different types of cancers to understand their correlation with prognosis and outcome.

Apart from the common, important biomarkers, there are additional markers that are unique to specific cancers, many of which have already been translated into clinical practice. The *HER-2/neu* proto-oncogene located on chromosome 17q21 encodes a transmembrane protein of the epidermal growth factor family[19] which is over-expressed in tumours of the breast,[20] prostate[21] and ovary.[22] More importantly, it is now the target of the newly developed drug trastuzumab (Herceptin; Genentech Inc., California USA), a specifically targeted monoclonal antibody that can synergistically act with concomitant chemotherapy in *HER-2/neu*-expressing breast tumours.[23] In the case of neuroblastomas, amplification of the *N-myc* gene can lead to over-production of the corresponding protein,[24] which can be an indicator of increased tumour aggressiveness, and this biomarker has now been adopted in the diagnostic and staging criteria.[25] Antithetically, in oligodendroglial tumours, the loss of heterozygosity on chromosomes 1p and 19q has been correlated with increased chemosensitivity and good prognosis.[26]

PROGNOSTIC MARKERS IN CANCER

The recent understanding of the molecular basis of cancer necessitates a coordinated use of various immunological and molecular

techniques with existing histopathological approaches towards the detection of specific cancer subtypes, their accurate prognostic staging and determination of appropriate therapeutic regimens. There is an ever-increasing body of literature that looks into the various molecular markers associated with different subtypes of cancers, but the importance of studying their combined effects and interplay is gaining prominence only recently. The following sections provide an overview of some of the major studies that have been carried out on various molecular determinants of specific cancers that have a relatively high epidemiological importance for the Indian subcontinent. It should be noted that similar, extensive studies have also been carried out in other cancers including oesophageal, prostate and ovarian carcinomas, as well as on melanomas, lymphomas and leukaemias, and have been reviewed elsewhere.

Lung cancer

The International Agency for Research on Cancer and WHO label lung cancer as the leading cause of mortality among men in India.[27] WHO histologically classifies lung cancer into the major subtypes of non-small cell lung cancer (NSCLC), squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinoma, which represent about 75% of all lung cancer cases.[28] The existing TNM staging for lung cancers does not, by itself, provide a wide distinction between the survival rates among the different tumour stages. Naruke *et al*. noted that in the clinical staging, there were no notable differences in the 5-year survival rates between stages IB and IIA, stages IIA and IIB, and stages IIIB and IV.[29] However, such discrepancies can impact the therapy that a patient receives, thereby affecting the overall prognosis. A multivariate analysis of 244 patients with stage I NSCLC from Harvard University showed p53 expression and absence of p21 expression to be significant predictors of recurrence, and the authors suggested the formulation of a pathological molecular substaging system in addition to the existing TNM system to better stage the tumours.[30]

p53 has probably been the most extensively investigated marker for lung cancer. A study of 114 cases of stages I and II AC and SCC for p53 accumulation showed that its increased expression in primary tumours and regional lymph nodes correlated with a more aggressive disease.[31] Studies on p53 for the detection of NSCLC have also shown a significant correlation with poor prognosis, thereby indicating the inclusion of p53 mutation detection in the work-up of NSCLC patients to employ more appropriate therapeutic strategies.[32,33] A combined analysis of mutated p53 and p21 expression in NSCLC revealed that patients with p53- and p21-negative tumours had the longest survival among those with different p53 and p21 features, suggesting a combined measurement of both in the prediction of prognosis.[34] Altered levels of pRb protein has been shown to be an independent prognostic marker for decreased overall survival in early-stage NSCLC,[35] while a combined study of p53, p21 and pRb have shown their value in categorizing NSCLC patients into distinct prognostic groups.[36] A study by Fontanini *et al*. has also shown that the anti-apoptotic marker Bcl-2 is significantly lower in patients who developed metastasis and is inversely related to p53 expression in NSCLC tumours.[37] The *BCL2* gene has been shown to promote tumour invasion and lung metastasis by inducing expression of the *MMP2* gene,[38] and IHC for MMP-2 and MMP-9 have been suggested to provide the best prognostic value for lung carcinoma.[39] Multivariate analysis has shown the expression of TSP-1 to be an independent indicator of better prognosis in NSCLC[40] and the pro-angiogenic factor VEGF to be a consistently poor prognostic indicator, either independently[41] or in combination with p53 or proliferative index.[42]

Breast cancer

Cancers of the breast have the second highest incidence, prevalence and mortality rates among all cancers in women in India.[27] The traditional modes of staging for breast cancer rely on histopathological reports and the number of axillary lymph nodes involved in determining the local stage of the disease. However, the broad spectra of pathological presentation of breast carcinomas themselves offer an almost intuitive hint on the varied molecular players that might be involved in the progression of the disease. Interestingly, the sex hormone receptor status is a very weak prognosticator of disease outcome but a strong prognostic marker of therapeutic response.[43,44]

Proliferation rates in breast cancer are associated with poor prognosis[44] and hence, the markers associated with increased cell proliferation and inhibition of apoptosis have been studied in great detail. A recent study analysing the transcript profiles of 251 p53-sequenced primary breast tumours pointed out that attenuated p53 transcript levels are associated with poorer patient survival, thus indicating the primary importance of a functional p53 status in predicting clinical breast cancer behaviour.[45] p53 positivity has also been associated with younger age and decreased 5-year progression-free and overall survival rates in inflammatory breast cancer.[46] Peters *et al*. have demonstrated a lower p21 expression in malignant stage I or II breast tumours compared to patients with benign breast pathology.[47] However, a more interesting report suggests that cytoplasmic localization of p21 is highly correlated with over-expression of phospho-p21 (at threonine 145) which in turn is associated with high expression of HER-2/neu and phospho-Akt. This triad is associated with worse overall survival.[48] An Italian study examining 153 primary breast carcinomas demonstrated that pRb scores correlated strongly with proliferation activity, and loss of pRb immunostaining characterized a more aggressive tumour phenotype.[49] Cell cycle regulatory proteins have thus been shown to be strongly associated with increased proliferative capacity and poor prognosis in breast cancers. In the apoptotic pathway, Bcl-2 has been associated with hormonal receptor status[50] and inversely correlated with p53 status in primary breast tumour specimens.[51] Reduced expression levels of Bax and Bcl-2, the prominent apoptotic markers, have been independently associated with lymph node metastasis in invasive breast cancers.[52] Human breast tumours have been demonstrated to become resistant to stromal TSP-1 *in vivo* by increasing the production of VEGF in the tumour cells themselves.[53] A loss of stromal TSP-1 expression in ductal carcinoma *in situ* of the breast is associated with a more histologically aggressive phenotype[54] while increased total VEGF positivity is a significant prognostic indicator for oestrogen receptor-positive breast tumours.[55] Separate studies have shown the prognostic values of MMP-2[56] and MMP-9[57] in breast cancer and the expression of both have been partly related to the expression of transcription factor activator protein-2 and *HER-2* oncogene.[58]

Colorectal cancers

With over 33 000 new cases being detected each year in India alone, colorectal cancers continue to be a major cause of morbidity and mortality.[27] Colon cancers are traditionally classified by Duke's staging, which is one of the oldest tumour staging systems. The system has been frequently updated through the past century,

though it has not been seriously challenged by any molecular system of classification so far. However, as with other cancers, colorectal carcinomas are also characterized by distinct molecular markers that are significantly associated with its prognosis. A review of the non-invasive methods of testing for colorectal cancers by Ouyang *et al*. notes that while faecal occult blood testing is a simple and inexpensive procedure that can reduce mortality from colorectal cancer, faecal DNA testing provides enhanced sensitivity for detection of the disease, although high costs restrict its use for general screening.[59]

The site of the tumour in colon carcinomas plays a very important role in the determination of prognosis at a morphological and molecular level. In a recent landmark publication under the *TP53* Colorectal Cancer International Collaborative study, mutations in the *TP53* gene were analysed in 3583 patients with colorectal cancer;[60] 34% of proximal colon tumours showed mutated *TP53* with increased lymphatic invasion, while distal colon and rectal tumours showed 45% mutations that corresponded with a worse survival. Mutations in exon 5 of the *TP53* gene were correlated with overall survival in proximal colon tumours. Reduced nuclear localization of p21 by IHC has also been shown to correlate with a poorer clinical course, decreased disease-free survival and increased incidence of distant metastases.[61,62] The association between pRb expression and outcome in colorectal carcinomas has not been extensively studied. The only important study that examined the relationship between the expression of pRb and p16 in 117 colorectal carcinoma patients who underwent curative resection with radical lymphadenectomy showed that aberrant expression of pRb and p16 independently affected post-resection survival, while their coincident abnormalities indicated the worst patient survival.[63] A multivariate analysis of a large study on colorectal cancer from the UK revealed an inverse correlation between p53 and Bcl-2, with a p53–/Bcl-2+ phenotype being significantly associated with a good prognosis, thus demonstrating its potential for providing stage-independent prognostic information in colorectal cancer.[64] Several studies have also shown a strong association between TSP-1– and/or VEGF+ tumours, and their tendency to demonstrate increased angiogenesis, and decreased recurrence and overall survival.[65–69] A broad multivariate study covering all classes of MMPs and their tissue inhibitors (TIMPs) has identified the MMP/TIMP profile as an independent indicator of poor prognosis in colorectal cancer.[70] MMP-2 and MMP-9 have been shown to be raised in colorectal carcinoma[71] and the balance of MMP-9 with its regulator RECK serves as a useful prognosticator.[72]

A special mention needs to be made about the use of molecular diagnostics in the screening, identification and prognostication of patients with hereditary non-polyposis colorectal carcinoma, an autosomal dominant syndrome accounting for 5%–10% of all colorectal cancer cases. A subset of these patients is characterized by mutation in one of two DNA mismatch repair (MMR) genes and high-frequency microsatellite instability, a condition known as Lynch syndrome.[73] Detection of the presence of microsatellite instability and the absence of MMR protein expression by IHC are suggested tools to identify individuals at risk for having Lynch syndrome, which has a comparatively early onset, better prognosis than that for the sporadic form of colorectal cancer, and an increased risk for cancer development in certain extracolonic sites.[73,74]

Bladder cancer

With close to 15 500 new cases detected each year, and almost 10 000 deaths, bladder cancer continues to be a major cause of cancer mortality in India.[27] Staging of bladder cancer is done using the TNM staging system, but it currently relies heavily on the proper sampling of tissues. An independent review of 217 cases of urothelial carcinoma (UC) suggested that portions of the muscularis propria were absent from samples of histologically documented UCs in up to 51% of cases, leading to interpretive discrepancies.[75] This observation strongly makes a case for having more objective modes of classifying UCs which can lead to a more accurate staging, thereby rationalizing the therapy offered. Our group at the University of Southern California is involved in carrying out extensive research on the prognostic value of various molecular markers for UC.

As with lung cancer, the *TP53* tumour suppressor gene is perhaps the most extensively researched marker for UC. Our studies have suggested a strong correlation between nuclear accumulation of p53 and a significantly increased risk of recurrence and death in UC, independently of the tumour grade, stage and lymph node status.[9] The multivariable analysis in this study stratified by grade, pathological stage and lymph node status has shown p53 status to be an independent predictor (and the only independent predictor in organ-confined UC) of recurrence and overall survival. Our studies have also shown that loss of p21 expression is an independent prognosticator of UC progression, whereas a positive p21 expression appears to negate the deleterious effects of p53 alterations on UC progression.[76] Other studies have also suggested that a p53- and p21-positive status puts patients with bladder carcinoma *in situ* at the greatest risk of recurrence, progression and mortality.[77] While investigations have predictably reported the involvement of retinoblastoma gene mutations in superficial and invasive UC,[78] our results have indicated that UC cases with undetectable and high pRb reactivity have identical recurrence and lower survival rates compared to cases with moderate reactivity, indicating that elevated expression levels may reflect an altered pRb pathway.[79] The study also showed that patients with altered p53 and pRb had significantly increased rates of recurrence compared to those with no alterations in either of the markers, while patients with only one alteration showed intermediate rates of recurrence. We have suggested that the altered pathway leading to pRb inactivation is through its hyperphosphorylation, which results due to loss of p16 expression and/or cyclin D1 over-expression.[80]

A relatively recent study published by our group at the University of Southern California analyses the combined effects of p53, p21 and pRb expression on the progression of UC.[81] The investigations revealed 5-year recurrence and survival rates to be 23% and 70%, respectively for tumours where none of the markers were altered in contrast to corresponding rates of 93% and 8%, respectively for tumours that showed an alteration in all the three markers. This landmark study in UC showed how a combination of markers across different pathways could provide more specific prognostic information than a single marker alone. *In vitro* studies on bladder cancer cell lines have shown Bcl-2 expression to interfere with the therapeutic effects of cisplatin and adenoviral-mediated p53 gene transfer.[82] Over-expression of Bcl-2 in UC has also been correlated with poor outcome in UC patients who have received synchronous chemoradiotherapy[83] or curative radiotherapy.[84] A study from the University of Southern California has also proved the significance of TSP-1 as an independent predictor of disease recurrence and overall survival after stratifying for tumour stage, lymph node status and histological grade, but is not independent of the p53 status.[85] TSP-1 expression was significantly associated with p53 expression and microvessel density

counts. Intriguingly, VEGF expression in superficial tumours has been shown to be 4-fold higher than in invasive tumours and 10-fold higher than in normal bladder tissues, thus suggesting the potential use of VEGF as an informative marker for early bladder cancer.[86] Papathoma *et al*. have correlated increasing levels of MMP-2 and MMP-9 with a statistically significant increase in tumour grade and invasiveness, thus suggesting their role in the degradation of the extracellular matrix of the basement membrane, thereby increasing the invasive potential of the tumour.[87]

USING A PANEL OF MARKERS

The above studies across various tumour types indicate that while there is limited value conferred by single determinants of prognosis for any given tumour, there is enhanced clinical utility when specific multiple markers are combined into panels. This is inherent in the molecular complexity of tumorigenesis and tumour progression, suggesting that modern medicine needs to account for the spectra of changes at the molecular level to understand the progression of cancers. However, in the enthusiastic effort of including a wide array of markers constituting any such panel, researchers and clinicians must be cognizant of certain pitfalls. While a review of high-impact literature can be an invaluable first step in drafting such a panel for any individual tumour, the practicalities of accurate diagnosis and overall prognosis need to be considered as well. For patients presenting with unknown primaries, one can see the obvious drawback of having tumour-specific multimarker panels. Therefore, the first step is the generation of a screening panel for carcinomas and sarcomas that can give an indication of their probable site of origin and molecular aggressiveness (which can be correlated with tumour grade on histopathology). This can be followed up with the use of tumour-specific marker panels that can have a combination of general tumour markers as well as site- and pathogenesis-specific molecular indicators.

The generation of such multimarker panels also needs to take into account the number of determinants that will be used in a single set. This logic is intimately linked with the resulting complexity of the panel that has a direct bearing on the time and cost involved in the generation of results, and the level of expertise required in interpreting them. In the Indian context, physicians will also need to take into account the ready availability of the required technologies for generation and interpretation of such data across the varied physical, cultural and socioeconomic milieus of the country. The ultimate goal will be towards standardization of such panels across departments, institutions and regions such that data exchange, analysis, storage and retrieval for any given patient can be performed unhindered in a practical manner.

Cancer-specific panels will need to be catered towards oncopathologists and treating physicians. A broad prognostic panel will cater to the need of the former group wherein general and tumour-specific markers can be incorporated in a rational and statistically appropriate way. Such a panel will have the power to predict the prognosis of the patient with the given molecular profile at a given point in time. Treating physicians will probably be more inclined towards a therapeutic panel, wherein a lesser number of genes can be used in a tumour-specific manner to judge parameters such as initial aggressiveness at the start of treatment, and the utility of the panel to monitor the therapeutic response. A judicious combination of both the suggested marker panels could then be used synergistically towards a more effective and personalized management of each patient.

METHODS FOR EXPRESSION PROFILING

Although cancers arise through inherited genetic mutations and/or acquired mutations over time, such mutated genes may transcribe into abnormal mRNAs which then translate into altered proteins incapable of normal cellular function, which ultimately lead to uncontrolled growth. Thus, it is crucial to not only examine for genetic mutations, but also the gene expression profiles and protein profiles. Recent technological advancements now allow bench-level profiling of cancer cells with the hope of bringing them to the bedside in the near future. Figure 2 schematically represents the potential impact of profiling technologies on the clinical management of cancer.

Transcript expression profiling

Expression microarrays are capable of generating vast databases of information that provide high resolution snapshots of cellular activity. Each experiment using expression arrays can be categorized into four separate processes: (i) array fabrication, (ii) sample preparation (RNA isolation from target populations) and labelling, (iii) hybridization of a labelled sample with immobilized probes on the array surface, and (iv) detection and data analysis. Arrays can be fabricated by two different methods. The flexible, in-house, customizable method uses a robotic spotter to print cDNA clones or oligonucleotides available commercially on solid surfaces such as nylon or glass. However, the spotted array has the disadvantages of requiring accurate annotations and the risk of cross-contamination.[88] As opposed to spotting synthesized probes on the surface, the alternative method uses technology that allows for oligonucleotides typically comprising 20–50 nucleotides to be directly synthesized *in situ* on the support. The *in situ*-synthesized arrays have better coverage and consistency; however, the cost of arrays and consumables can be relatively high.[89] mRNA isolated from cell lysates can be amplified and coupled with fluorescent, chemiluminescent or radioactive labels followed by detection using an appropriate scanner after hybridization with the immobilized probes on the array surface. Array-based expression technologies have become very powerful and popular high-throughput tools for studying gene expression profiles and their usage can be reflected in the exponential increase in the number of publications in the past few years.[90–92]

Although the array technology has potential for large-scale measurement of all human genes simultaneously, in its current form, at least 1 μg of RNA is required for each experiment.[93] Willey *et al*. have developed a modified quantitative method for competitive reverse transcription–polymerase chain reaction (qc RT-PCR) that allows the simultaneous measurement of many genes using nanogram amounts of cDNA.[94,95] The transcript levels are expressed as numerical values per million molecules of a housekeeping gene such as b-actin, thus allowing standardized intra- and intersample comparisons.

Protein expression profiling

Two-dimensional gel electrophoresis (2DGE) is a widely used and powerful technique for the isolation of biomolecules for further characterization. More recently, it has been applied for the identification of potential biomarkers for the diagnosis of cancer. Generally, proteins in a 2DGE are separated by their isoelectric points in the first dimension followed by separation based on their molecular masses. Visualization of hundreds of biomolecules on a single gel can be accomplished by radioactive or fluorescent labelling. Differences in protein expression are quantified by

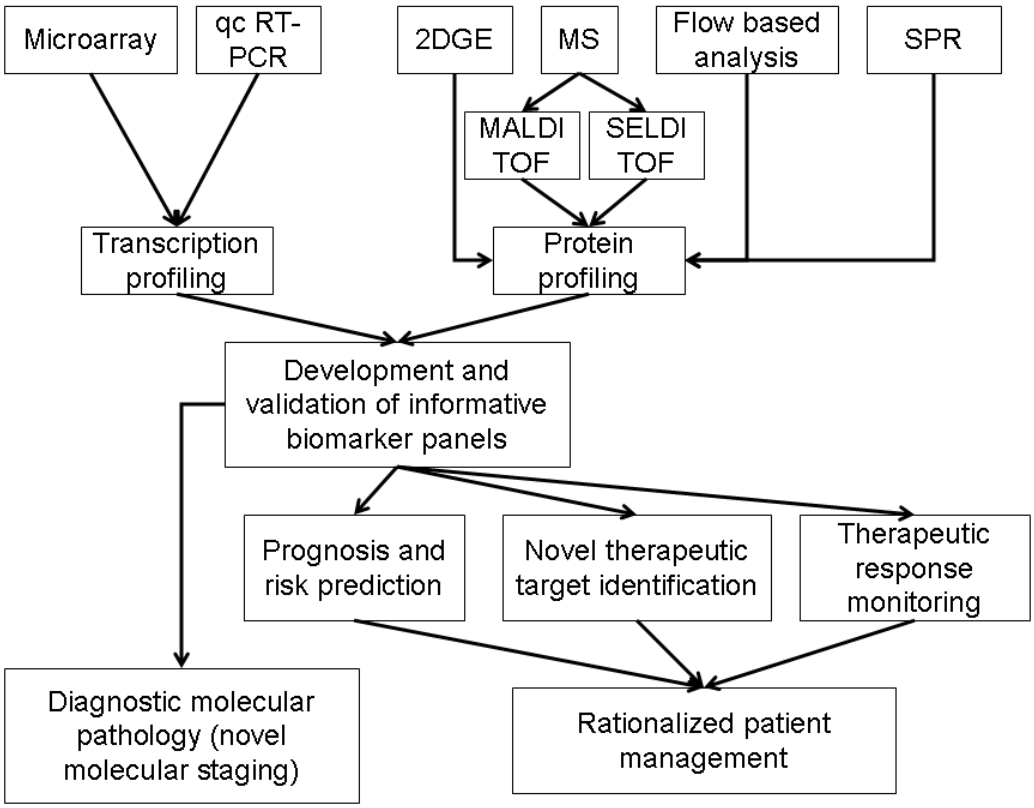

FIG 2. Impact of biomarker profiling on cancer management. qc RT-PCR quantitative, competitive reverse transcription–polymerase chain reaction 2DGE 2-dimensional gel electrophoresis MS mass spectrometry MALDI matrix-assisted laser desorption ionization TOF time of flight SELDI surface-enhanced laser desorption ionization

comparing the ratio of intensities between spots from independent gels. It has been shown that 2DGE has the power of resolving over 1000 proteins as individual spots;[96] however, this method is not suitable for large-scale clinical testing because it is labour-intensive, time-consuming, has high sample consumption and is difficult to standardize across laboratories. This method is incapable of detecting molecules that are very small or very large, highly acidic or highly basic, or if they are in low abundance.[97] Moreover, it only separates the proteins and does not provide sufficient information for identification of the spot, which requires further downstream processing.

Time of flight mass spectrometry (TOF-MS) is a method to identify proteins by estimating the mass-to-charge ratio through the measurement of velocity (time of flight for an ionized peptide to traverse the path between a protein-adsorbed metal plate surface and the detector).[98] Matrix-assisted laser desorption ionization (MALDI) and surface-enhanced laser desorption ionization (SELDI), techniques that are capable of analysing high molecular weight proteins (>100 kDa) and small peptides (~30–300 Da), respectively, enable the conversion of biomolecules into a charged state that is essential for analysis by TOF-MS. In MALDI, the procedure involves precipitation of the sample molecules with an excess of a matrix material such as a-cyano-4-hydroxycinnamic acid or dihydroxybenzoic acid, which has an absorbance at the wavelength of the laser. The precipitated solids are then irradiated with laser pulses and the matrix material imparts energy to the biomolecules, which are subjected to a process of desorption and ionization followed by analysis using MS to measure the mass-to-charge ratio of the protein, peptide or peptide fragments based on TOF. Coupled with the separation power of 2DGE, MALDI-MS is a very powerful tool for discovering possible biomarkers for cancer.[96] SELDI is an affinity-based MS method in which proteins are selectively adsorbed to a chemically modified surface that permits binding based on hydrophilic, ionic or hydrophobic interactions. The proteins are identified by laser desorption TOF mass analysis. SELDI has become a tool of choice for proteomics in recent years due to its versatility, speed and ease of use in sample preparations while being highly reproducible (<10% variation between runs) and sensitive (detection limit ~femtomolar).[99] A potential clinical application of the SELDI system is towards the development of immunoassays by immobilizing an antibody to a specific biomarker on the array surface. For example, Xiao *et al*. have successfully quantified the prostate-specific membrane antigen from serum for the diagnosis of prostate cancer using SELDI immunoassay.[100] Apart from detecting a single biomarker, SELDI is also capable of detecting a pattern of proteins for cancer diagnosis. In a landmark study, raw, unfractionated sera from patients with ovarian cancer was analysed by SELDI to produce a TOF bar code consisting of thousands of protein ion signatures for diagnosis.[101] However, MS continues to be an expensive method that requires highly skilled technical expertise.

Surface plasmon resonance (SPR) spectroscopy is a technique capable of monitoring surface-to-molecule binding in real time and without labels. Diverse biomolecules, including membrane-bound and serum proteins, nucleic acids and lipids, have been examined with SPR. The first component of the interaction to be studied is immobilized covalently (via NHS-esters, amine-, hydrazine- and sulphydryl-functional groups)[102] to the hydrogel or carboxylated dextran interface matrix, and the other interactants are passed over the chip in solution. The change of concentration at the sensor surface, reflecting the progress of the interaction studied, is monitored in real time by optically measuring the change in the refractive index and an SPR response is detected. The technique does not require molecular labels for detection and can measure mass changes as low as 10 pg/mm.[89,103] Clinical applicability of this method has been shown using the BIACORE 2000 system with a specific antibody bound on the surface to monitor the development of infusion-related adverse events in patients on a clinical immunotherapy trial for colon cancer, where the levels of antihuman antibodies in serum were examined, thereby allowing patients to be removed from the study before the onset of severe infusion-related adverse events.[104]

Luminex assays use polystyrene microspheres internally dyed with differing ratios of two spectrally distinct fluorophores to create a family of 100 differentially spectrally addressed bead sets. Each of the 100 spectrally addressed bead sets can be conjugated with a capture antibody specific for a unique target protein. In a multiplexed assay, such antibody-conjugated beads are allowed to react with the sample under investigation (plasma, serum or cell culture supernatant). After washing, secondary or detection antibodies are added to the microtitre plate well to form a capture sandwich immunoassay. The assay solution is analysed by the fluorometric array reader, which obtains two fluorescence readings for every single bead: one that identifies a bead as a member of one of the 100 possible sets, and another that measures the amount of fluorescent dye, phycoerythrin (PE), bound to the detection antibody in the assay. The amount of green fluorescence (from PE) is proportional to the amount of analyte captured in the immunoassay. Using this assay, thousands of beads can be analysed in seconds, allowing up to 100 analytes to be measured in a 96-well microplate in one hour.[105] In addition, since the fluorescence from each bead is measured independently, sufficient data points are accumulated to allow for assaying each sample in a single well and not in duplicates.

IMPLICATIONS FOR PATIENT MANAGEMENT

Employment of a molecular marker panel for the detection, prognostication and therapeutic planning of cancers is now moving towards reality with the availability of sophisticated high-throughput techniques that can accurately profile multiple determinants simultaneously. Outputs for the marker panel can then be run through statistical or genetic programming algorithms to generate classifier signatures that can then objectively and precisely group a tumour sample to a particular class.[106]

With an increase in the number of specialty centres devoted to cancer treatment and with a growing realization of the need to revise tumour staging based on more accurate prognostic and therapeutic indicators in the form of molecular markers, there is an urgent need to revamp core pathology and diagnostic facilities at such centres to perform molecular profiling. These departments can then serve to provide pathologists and clinicians with more objective and precise methods of cancer staging and treatment classification. Such facilities can provide diagnostic and therapeutic monitoring services using common platforms, thus proving to be comprehensive centres for patient management, from detection to intervention and beyond. The computing backbone at such cores can also liaise with medical records departments at hospitals to combine diagnostic and therapeutic monitoring information for the same patient to ensure better follow up. They can also serve as large-scale repositories for patient data that can be an invaluable resource for retrospective, population-based and epidemiological studies. The availability of such data in electronic format can also simplify the secure transmission of critical patient information between departments, physicians and institutions.

While the revised staging of cancers based on their molecular profiles can serve as an efficient tool to address clinical problems currently facing oncopathology, molecular marker profiling in cancer can go beyond the scope of clinical research via the combined advantages of early detection, risk prediction, drug target discovery, rational administration of therapies and effective therapeutic monitoring. It can be hoped that biomarker profiling can directly impact cancer patient management by significantly reducing morbidity and mortality.

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