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Cancer Proteomics: How Far are We from the Clinics?

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ABSTRACT Cancer, in spite of several years of research and consequent improvements in clinical management of the disease, shows limited decline in the associated patient mortality and morbidity. In order to achieve better and early diagnosis, design appropriate treatment strategies and precise prognostication, molecular markers which could subclassify tumors are being assessed to complement TNM classification. Plethora of information is spilling out from the recent high-throughput technologies for global profiling. Time has come to assess the technologies, study designs and data processing to obtain information which is clinically relevant. This review analyses the state of the art in Cancer Proteomics and the issues, which need to be addressed to move the technology from the bench to the bedside.

Cancer Proteomics is the application of proteomics technology to identify qualitative and quantitative differences in proteins detected in the tumor tissue or other relevant body fluids of a patient with cancer versus those in healthy individuals, to aid in cancer diagnosis, prognostication and treatment more effectively.

Conventional Clinical Classification and Need for Markers

Cancer, in spite of several years of research and consequent improvements in clinical management of the disease, shows limited decline in the associated patient mortality and morbidity. *Several reasons limit the success of strategies for cancer management. One* and very important is that it is a heterogenous disease. Even within a single cancer site, tumors have markedly different behaviours and are associated with widely varying prognoses. Till date the most widely used guideline for prognostication in cancer medicine has been the *TNM staging*, which is based on anatomical extent of the disease. It provides a basis for prediction of survival, choice of initial treatment and stratification of patients in clinical trials (www.cancerstaging. org).

In recent years, molecular markers which could further subclassify tumors have been assessed to complement TNM classification to bring precision to prognostication (Ludwig and Weinstein 2005). *Secondly*, the heterogeneity of disease fails a common treatment regimen. Even in a given patient, the genetic instability of invasive cancer means that combination of therapeutic agents will probably be required to prevent a cell from developing resistance to the targeted therapeutics. Thus *additional targets for therapy need to be identified* (Maitland et al. 2006; Corchero and Fernandes-Salguero 2005; Manne et al. 2005). *Lastly*, most people who develop cancer have advanced disease at the time of diagnosis. Survival rates for people diagnosed with advanced cancer have changed little over the past 20 years. At this time, therapeutic modalities are very limited in their success and most of treatment is spent providing palliative quality of life therapy. By contrast, survival is relatively good when these cancers are diagnosed at an early stage. *Tools for early detection are therefore necessary* (Sciubba 2001; Hirsch et al 2002).

Analysis of *molecular alterations*, in DNA, RNA or proteins in cells undergoing malignant transformation have increasingly revealed individual molecules that are associated with malignant transformation, some of them have been shown to be related to tumor stage and grading or may be indicative for the prognosis and the clinical course of the disease. They cover molecules associated with various aspects of tumor biology such as cell cycle control (Colozza et al 2005), apoptosis (Thomadaki et al. 2006), matrix degradation, adhesion molecules and immunologic tumor defense (Chin et al. 2005; Whiteside 2004). US Food and Drug Adminis-

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tration has approved several clinically relevant biomarkers-mainly proteins, such as human chorionic gonadotropin β for staging of testicular carcinomas and expression of oestrogen and progesterone receptors for selection of hormonal therapy for breast cancer (Ludwig and Weinstein 2005). Chromosomal markers have found application in diagnosis of leukemias (Kim 2004), bladder cancer and breast cancer (Ludwig and Weinstein 2005).

In the clinics, *these molecular biomarkers* are supplementing the already existing tools for cancer diagnosis and disease management. Their utility is however limited by several factors. Conceptually, from the point of view of tumorigenesis, the cancer phenotype results from the sum total of the effects of all the numerous contributing transformed loci. Over two decades of investigations have shown that an alteration in many different genes, each with an allelic variation, contributes to the total observed variability and heterogeneity, which is impossible to assess by studying single markers. This is reflected in lack of absolute predictability for a single or a set of markers (Caprioli 2005). When their expression is tested in biological samples, a grey zone exists for samples, which fall in the zone overlapping the expression in control and the test, wherein decision-making is difficult (Baker 2005).

More recently, *battery of biomarkers* are being increasingly viewed as more accurate than individual biomarkers (Mitra et al. 2005). Biostatisticians have long known that clusters of variables can be used to differentiate two sets. Co-expression of a combination of these markers have been successfully applied to stratify patients. For examples USFDA has approved oncotype Dx test, which assesses tumor expression of 21 genes, to predict potential of breast tumors to recur and respond to therapy (Baker 2005). More recently, the cytochrome P 450 genotyping test to assess drug metabolism has become available (Hanash 2004). Moreover, technologies established for global profiling of macromolecues like CGH, microarray and proteomic analysis have *eliminated the need for prior knowledge* of markers or confounding of results by biased selection of known markers. For example in a microarray set up, expression of thousands of genes can be assessed in a single experiment, making the matrix of markers large enough to allow subclassification (Dahl et al. 2006; Wong et al. 2006; Kim 2004). During the past decade, genomic microarrays have been applied with great success to the molecular profiling of tumours, which has resulted in a much more detailed classification scheme as well as in the identification of potential gene signature sets (Mayr et al. 2006; Patil et al. 2005).

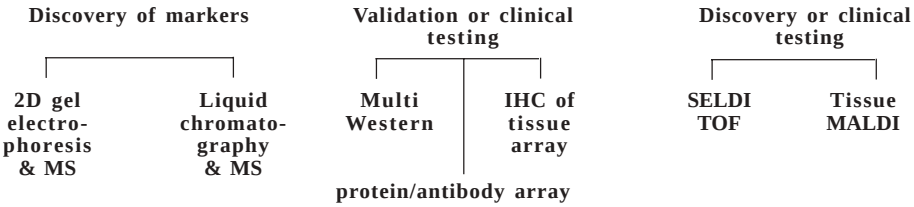
In the post-genomics era, the *significance of protein profiling* for deciphering functional pathways in cell physiology and their aberrations in pathology is widely acknowledged and receiving immense attention for early detection, diagnosis and prognosis. The key proteins driving oncogenesis undergo translation and post-translational modifications, which are not reflected in their transcription or genomic profiles. Since proteins are the most effective drug targets, their profiling and understanding of the functional pathways altered in tumor would provide additional or new targets for therapy. Moreover, when response to therapy is to be assessed, it will be necessary to analyze proteins in readily accessible serum samples and other body fluids, since repeat biopsies are not a realistic option. Therefore, if individualization of therapy is to move into the practical world, blood-based assays, serum and body fluid proteomics become critical (Abramovitz and Leyland – Jones 2006). In this context, the utility of immunoproteomics to analyse autoantibody response in patients with cancer is an area under intense investigation (Anderson and LaBaer 2005; Casiano et al 2006).

Proteomic Technologies useful in Clinical Cancer Research

Global profiling of proteins in tissues and body fluids has been limited by the number of proteins, out of the 40,000 expressed by the genome that could be separated and detected by the present technology. On-going technological improvisations are trying to meet this challenge.

Several new technologies are available and emerging to either discover combinatorial biomarkers or validate the known as well as new biomarkers in a translational mode. They are reviewed in detail (Misek et al. 2004; Conrads et al. 2004; Lim and Elenitoba-Johnson 2004; Srinivas et al. 2001; Nair et al. 2004; Petricoin et al. 2004) and are summarized below based on the concept of application.

Biological mass spectrometry (MS) has revolutionized the field of protein profiling.



Variety of instruments with distinct resolution, sensitivity and mass accuracy are now available which can identify proteins by peptide mass fingerprinting or de-novo sequencing (Solassol et al. 2006) .

Analysis of *protein mixtures* like tissue lysates and body fluids, requires their *prior separation* to reduce the complexity. These methods separate proteins on the basis of charge and/or molecular weight. A commonly used method is two dimensional gel electrophoresis. Sensitivity of detection in *2D electrophoresis* is limited by that of separation and staining by the practically achievable dimensions of the gel, limiting their use for the detection of mostly high abundance proteins in a non-prefractionated sample. Comparison of spots in control and test gels even with the available softwares have practical limitations which has not improved greatly in the co-electrophoresed test and control samples labeled differentially with fluorescent dyes (Ornstein and Petricoin 2004). Another method of protein separation is *liquid chromatography,* in which column with properties to bind protein/peptides with specific characteristics are employed singly or in combination to separate highly complex protein mixtures. This method is useful for separation of low-abundance proteins (Frohlich and Arnold 2006). The differentiators in control and test samples could be analysed in a single run using the differential labeling techniques like ICAT (Turecek 2002) as well as ITRAQ (Aggrawal et al. 2006)

Separation of proteins can also be achieved in a mass spectrometry system with the SELDI technology wherein protein chips are available for a variety of different chromatographic surfaces that are designed to retain specific classes of proteins based on physicochemical properties such as hydrophobicity and charge. The approach has found wide application in clinical proteomics but of late has also received criticism as a technique (Ardekani et al 2002; Engwegen et al. 2006). SELDI analysis of peripheral blood plasma has generated a molecular profile that gives a strong positive prediction of recurrence in ALL (Albitar et al. 2006) as also prognostic markers for node-negative breast cancer patients (Ricolleau et al 2006)

Proteins can be detected in samples individually or in combinations with *simpler methodologies*, provided specific antibody/ antibodies are available for each protein. This approach is being used for validation of markers or for developing clinical tests that are easy to perform in diagnostic laboratories using techniques such as multiwestern immunoblotting and antibody /protein arrays for the analysis of proteins in *tissue lysates* and *body fluids*. Antibody detection of molecules in *tissue sections* or in tissue arrays is achieved by *immunohistochemistry* (IHC). The Swedish Human Proteome Resource Project (www.hpr.se) with its target to generate antibodies for all annotated proteins will contribute immensely towards these efforts (Uhlen 2005). Beyond separated protein mixtures, *whole tissue MALDI* is now projected as the next sensitive technology that can be used in a clinical set up (Caldwell and Caprioli 2005).

Lot of data has emerged using the above mentioned proteomics technologies and statistics is being used to evaluate the co-expression patterns of differentiators for their biological relevance. To state a few representative examples, Dwek and Alaiya (2003), Alaiya et al. (2002), Hellman et al. (2004) have achieved segregation of normal tissue, benign epithelium and tumor tissue in breast, ovary and vagina respectively.

The potential of the proteomic approaches is apparently multifarious. However, the sensitivity and specificity of prediction needs further refinement of concept and technology. This is achievable if certain nuances *in experimental design and data analysis are considered* and given intense attention to address the lacunae.

Experimental Designs that Need Attention

The areas which need attention are the consideration of cancer as a *systemic disease*, the *reductionist approach* where necessary,

optimal selection of sample and sample size, appropriate statistical or computational analysis for the co-expression of markers, beyond quantitative and qualitative alterations into *altered localization*, both subcellular and within the tissue sample and molecular alterations such as *post-translational modifications*.

Cancer attains a *systemic disease* status as it advances. Clonal expansion of the transformed cell is a continuum of molecular alterations, exposed to the extracellular microenvironment. These molecules themselves or microenvironment modified by them evoke responses in the neighbouring or immune cells which in turn facilitates tumor progression. Thus, the imprints of the progressing disease extends out into the microenvironment of the tumor-host interface, the surrounding stromal and vascular compartments and outwards, to the circulation macroenvironment. While detection of markers directly in tissue biopsy would correlate with the primary disease, to get a *complete picture of the disease state it is essential to screen the tumor tissue, surrounding stroma, serum and other relevant body fluids for probable markers.* Such a systems approach has been elegantly reviewed for breast cancer by Abramovitz and Leyland-Jones (2006).

The challenges of this approach and meaningful analysis of complex data to yield specificity, has on the other hand necessitated the use of *microdissected* tumor tissue to identify the contribution of the marker from each of the sites towards the trasformation process. Reports like detection of stathmin as a potential biomarker for both tumors of salivary gland (Nakashima et al. 2006) and oral squamous cell carcinoma (Kouzu et al. 2006) has necessitated confirmation of the finding in *microdissected tissue (*Rekhter and Chen 2001*)*.

Beyond the qualitative and quantitative analysis of tumor markers which contribute to the prognosis of the disease it has become essential to assess *the location of these markers within the tumor mass as well as its intracellular localization*. In particular, the invasive front of the tumor appears to be of great importance for prognostication as seen from the study by (Abramovitz and Leyland- Jones 2006) which shows that intratumoral rather than peritumoral dendritic cells are often indicative of survival. In colorectal carcinoma, tumor leading edge has shown Ki 67 expression in the tumor cells (Rubio 2006) while cathepsin B is predominantly expressed by macrophages at the leading edge of the invading tumors (McKerrow et al. 2000) . Such evaluations require investigations in several areas of the tumor tissue, necessitating larger representative tumour tissue samples rather than just punch biopsies. In this regard, the use of tissue arrays for analysis of different areas of the specimen needs careful consideration. Another factor that needs attention is the *intracellular localization* of the molecules and any alteration in the tumor tissue which could have prognostic significance. This is evident in a report wherein nuclear localization of GST Pi, in the tumor tissue, is useful in the prognostication of glioma (Ali-Osman et al. 1997).

The *posttranslational modification of proteins*, by phosphorylation, glycosylation, ubiquitinylation, proteolytic cleavage etc and sometimes consequential change in subcellular location, as a cause/consequence of transformation also needs attention. Petricoin et al (2005) have provided a vision for individualized combinatorial therapy based on proteomic mapping of phosphorylation end points in clinical tissue material.

Assuming that not only tumorigenesis and progression but also prognosis has a multifactorial molecular background, it is apparent that *larger samples sizes* are more likely to escape blurring of results due to uncontrolled effects and thereby help in reaching statistical signi-ficance. The low predictive value of those markers which fail to reach significance may therefore in part be accounted for by insufficient sample size (Schliephake 2003; Lin and Kibbe 2005). Further, sufficient sample size could underscore the prognostic relevance of markers identified by IHC, in spite of the semiquantitative nature of the analysis as shown by Lyall et al. (2006) in their study of colon cancers. In a traditional multivariate analysis, the ratio between sample size and number of variables is suggested to be larger than 30:1. With a small sample size and huge variable search space, the probability of finding associations by random chance is quite high even when analysed at statistically stringent conditions (Lin and Kibbe 2005). The utility of Fuzzy logic in these situations needs to be considered.

The expression level and molecular forms of proteins are a consequence of genomic factors, structural modifications, regulatory processes involving cellular integration and balance (or

imbalance), environmental factors, temporal processes etc. The net sum of these gives rise to a proteome expression level and distribution that reflects the integrated metabolic state of the cells in that tissue at any given point in time as the *proteome is dynamic*. (Nair et al. 2004). Therefore, assessment of biomarkers in repeat biopsies is necessary. This has practical limitations for biopsy material, but could be overcome in the analysis of markers in body fluids.

How Far are We from Clinic?

In the comparative proteomic approach for marker identification, significant problems remain. Most of the proteomics studies yield information about molecules expressed in large amounts, easily detected by the present technology. These, more often than not are useful for determining the transformed state and could be used in diagnosis. The molecules, which would be required for prognostication and/or are potential therapeutic targets are expressed in lower amounts. It is essential that the technology used for profiling is sensitive enough to detect the differentiators from both these categories.

Other parameters which can confound the results could be the identification of markers that reflect non-disease-specific systemic responses of the patient or other secondary to the disease. Several environmental factors such as diet and tobacco habits can lead to complex and rapidly changing protein patterns not directly related to the primary disease.

It is becoming apparent that genomic and proteomic technologies have the potential to discriminate between subtypes of tumors. Computational and mathematical techniques, such as multivariate analysis or machine learning, can pluck out differences or clusters of differences that distinguish members of one group from the other, at least for that particular sample set. However, given enough variables, some will discriminate the treatment group from control groups by chance, with often misleadingly impressive statistical significance. Caution is therefore necessary in these analyses and v*alidation of the sensitivity and specificity of markers* is obligatory. Biomarkers should be confirmed in independent studies across institutions before being used to make decisions and the level of certainty required varies with the biomarker.

Finally, to translate the validated battery of markers into a viable laboratory test, certain issues need to be addressed. The technological platforms used for global proteomic profiling such as mass spectrometry have limited reproducibility across laboratories. It is observed that variability attributable to instrumentation has been confounded by differences in sample collection, data processing and analysis methods. Thus, researchers view these technologies as a rapid screening tool for generating profiles but difficult for routine screening application. It is essential to develop simpler detection kits for this battery of markers using low-throughput techniques, such as ELISA, multiwestern assays, protein arrays which are feasible to perform in clinical laboratories at an affordable price.

As diagnostic and prognostic markers need validation, precaution needs to be taken before a differentiator is declared as a molecular target for therapy. Although the biomarkers prove to be discriminators between disease states, that should not be extrapolated as the reflection of its role in the mechanism of disease and thus view it as a potential drug target. This requires integration of the information with relevant biology.

SUMMARY

Global profiling in general and *proteomics* in particular, has great potential for providing molecular signatures, which could be used for diagnosis, prognosis, for monitoring response to therapy as well as in identifying new therapeutic targets. The success at the bench is poised to be taken to the bedside with a lot more precision in analyses.

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