Review

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Identification of Therapeutic Targets for Cancer: Proteomic Technologies and Strategies are the Key to Success

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With the emergence of the field of 'omics' a new era of systematic global profiling of cellular molecules has been initiated in biology. Different 'omics' approaches have been extensively used to identify biomarkers for better diagnosis and prognosis, therapeutic strategies and monitoring response to therapy in diverse types of cancers. Proteomics is the approach of choice for identification of therapeutic targets. This is because therapeutic modulation of expression, post-translational modification and activity of a protein can directly rectify the derangement in the disease-causing cellular pathway. The current review scans literature on tumor proteomics to understand the influence of developments in proteomics technology and study approaches on identification of targets for therapy. Diversity of tumor types, molecular heterogeneity in pathologically indistinguishable tumors provides ample challenge to assess the strength of proteomics in identification of drug targets. The review highlights comparative proteomic profiling by gel-based or gel free approach, in tumor and normal tissues or chemo-resistant/sensitive tumor tissues have identified differentiator proteins, with potential as targetsas therapeutic targets. Further, along with evolution in proteomic technologies for identification and quantification of proteins, various tools for functional analysis of proteins have contributed to strategies for target identification. It also suggests that future advances in quantitative, functional and structural proteomics isare necessary to widen the search for therapeutic targets.

INTRODUCTION

The pace of development of technology is a rate determining factor in the rapid progress of basic and applied sciences. This is exemplified in the healthcare sector by the translational advances of the technological inventions in the healthcare sectorwhich include computerized tomography (CT) and magnetic resonance imaging (MRI) for diagnosis; laparoscopes and cardiopulmonary bypass pumps for surgery; and systems for therapies such as radiotherapy and dialysis. Apart from these technologies which are directly used in clinics,

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advances in optical and analytical instruments have revolutionized biomedical research contributing to the progress in understanding pathobiology of diseases and their management. The current review focuses on proteomic technology-driven advances in the identification of therapeutic targets in various cancers, as a milestone in development of targeted therapeutics.

Serendipitous discoveries of drugs and drug targets

Identification of novel therapeutic targets remains an area of interest to clinicians and biotechnology and pharmaceutical industries. Several drugs have been discovered earlier by extraction of the active principle from natural sources such as plants traditionally used to treat a disease (Cragg, 2013). A few drugs were discovered by serendipity, a classic example being penicillin from a contaminating fungus (Bennet, 2001) and a cancer chemotherapeutic vincristine by its undesired myelosuppressive effect when used to treat diabetes (Johnson, 1963). With improved understanding of molecular machinery of the cell and aberrations associated with disease conditions, efforts were made to design drugs to target the disease-causing molecule. This approach was favoured for diseases with an established causal association with a molecular alteration. For example, in chronic myeloid leukemia (CML), inhibition of the transforming tyrosine kinase has been an excellent targeted therapy in CML patients (Freireich, 2014). However, for a long time, serendipity remained the basis for discoveries of drugs as well as for the identification of targets as in CML. Such discoveries require fortuitous co-occurrence of the phenomenon which led to the discovery and an alert analyst. A structured approach is necessary for identification of therapeutic targets and discovery of new drugs.

'Omics' for systematic identification of molecular alterations in tumors

With emergence of 'Omics', biology began a new era of planned and systematic search for global molecular alterations in diseases. The strength of this approach is evident from its potential to unravel key molecular events from the numerous molecular alterations seen in cancer (Bertrand, 2015; Castro-Vega, 2015). Multifactorial and multigenic origin of cancer is reflected in the diverse molecular alterations in pathologically indistinguishable tumors, which progress and respond differentially to drugs and therefore require an individualized therapeutic approach. Interrogation of differences in genome, transciptome, proteome, metabolome, lipidome have led to the identification of several cancer biomarkers useful in diagnosis (Du, 2014; Liu, 2015), predict disease prognosis (Minca, 2014; Shipitsin, 2014), assist in choice of therapy (Fėnichel, 2014; Sjøholt,

2006) and monitor response to therapy (Rebecca, 2014).

Proteomics: The 'Omics' of choice for identification of drug targets

Proteins are functional molecules in a cell and alterations in their function can affect cell phenotype. Proteomics, a global study of proteins, is therefore an approach of choice to identify drug targets. The study of a cell proteome is challenging due to the complexity of protein structure; effect of post-translational processing i.e., cleavage or modification of function, sub-cellular localization and changes in interacting molecules including substrates (Parker, 2014); dynamic range of proteins expressed in a cell (Corthals, 2000) and the temporal variations in the proteins.all the above variables. In a disease state, alterations in any of these parameters may occur in one to several proteins. Challenges posed by these compounding factors to proteomic profiling have been addressed at every step of development of proteomic technology and supported by advances in the fields of genomics (Wang, 2014), bioinformatics (Boguski, 2003) and computational biology (Dowsey, 2003).

Technological advances meet the challenges of profiling complex proteomes

Protein identification by mass spectrometry

Emergence of the field of proteomics began with the changes improvisations in mass spectrometry (MS) instrumentation and techniques in late 1990s. Proteins and other biomolecules fragmented by the previously used ionization methods, were preserved intact by the softer ionizations – electrospray ionization (ESI) (Karas, 1988) and matrix assisted laser desorption ionization (MALDI) (Fenn, 1989) promoting MS as a tool for biologists. The ionizers were coupled to an analyzer, such as a quadrupole, time of flight, ion trap, etc. which separated the ionized molecules on the basis of their mass/charge (m/z) ratio. MS-based identification of proteins was achieved by comparing the masses of peptides generated by cleavage of a protein using specific protease and those generated 'in silico' by digestion of sequences available in public databases with the same protease, an application called peptide mass fingerprinting (PMF). In tandem mass spectrometry platforms (MS-MS) with more than one analyzer, peptides detected in the first analyzer are put through controlled fragmentation by collision induced dissociation (CID). The accurate masses of the peptide fragments obtained from analysis in the second analyzer, when processed by appropriate software designedgenerated the sequence of the peptide. The sequence is used for protein identification. Thus mass spectrometry circumvented the need for a probe to detect the protein of interest from a complex mixture of proteins (Abersold, 2003). Further, deeper interrogation of the proteome, essential for biomarker

discovery, was made possible by the features in mass spectrometer which allowed data-independent fragment analysis (Sajic, 2014). Further advancement in the fragmentation mechanisms, introduction of electron transfer dissociation (ETD), improved detectability of labile post-translational modifications in proteins (Kim, 2012). Advances in mass spectrometry therefore improved the detection, identification and knowledge of the post-translational modifications in proteins.

Reduction in protein complexity before mass spectrometry

The strength of mass spectrometry for protein identification is compromised in biological samples due to the complexity of proteome and dynamic range of protein expression. High or medium abundant species in a sample may interfere with the detection of low abundant species, called "suppression effect". Therefore to enable identification of less abundant proteins and to enrich less concentrated species, clinical proteomics studies require fractionation of proteins from complex mixtures, prior to proceeding for identification by mass spectrometry analysis. The strategy which can optimally reduce the complexity would differ in each sample (Camerini, 2015).

Among gel based separation methods, two dimensional gel electrophoresis (2DGE) has been the method of choice. Separated proteins are subject to in-gel digestion with specific proteases, peptides are extracted from the gel and subjected to MS for protein identification. The technique of 2DGE has metamorphosed due to availability of immobilized pH gradients (IPG), multi-gel electrophoresis apparatus improving the reproducibility of profiles, and staining protocols using fluorescent dyes with improved sensitivity and linearity over a wider dynamic concentration range. IPG strips are available in micro pH ranges for wider resolution, and thereby improving detection of proteins (Gorg, 2000). Apart from 2DGE, capillary electrophoresis and agarose gel isoelectric focusing are used to separate proteins from biological samples (Manabe, 2000).

Liquid chromatography (LC) is a versatile method for protein separation as different column chemistries allow separation of proteins based on distinct characteristics (Di Palma, 2012). Since ESI can ionize samples introduced in liquid phase, LC-MS platforms were designed, wherein sample separated on LC can be introduced directly into the ionizer of MS. However, as biological samples are obtained in limiting amounts, efforts have been made to increase the sensitivity of detection by tapping measures beyond the improvisations in the hardware of MS. In LC-MS systems, reducing the flow rate contributes to higher overall sensitivities due to the higher efficiencies in ESI and reduced ion suppression effects (Köcher, 2014). Nano LC-MS-MS is the

configuration of choice for biological samples analysis.

Separation and identification tools for differential quantification

Distinction between expression of proteins in disease and normal state requires quantitative evaluation of the expression. Several tools are available to achieve the same. Quantitative proteomics can be categorized into absolute and relative types. Absolute quantitation determines changes in protein expression in terms of an exact amount or concentration of each the protein present; whereas relative quantitation determines the up- or down-regulation of a protein relative to the control sample, generally used in clinical proteomics. In MS based quantitation, the relative concentration can be obtained by: Label free methods are based on less rigorous mass spectrometry, with more reliance on bioinformatics and separation techniques. Chemical labeling is applicable to a wider range of biological samples, and methods such as isotope coded affinity tags (iCAT) and isobaric tags for relative and absolute quantitation (iTRAQ), are favoured in quantitative proteomics. The control and test samples are labeled with separate tags, and intensity of the same ion with distinct tags indicates the relative quantitation (Elliott, 2009). Protein quantitation can be done by two dimensional difference gel electrophoresis (2D-DIGE) wherein proteins from different samples are labeled using dyes that provide different fluorescence wavelengths for detection. The labeled samples to be quantitated are mixed in equal proportion and separated on the same gel. The gels are scanned and the relative fluorescence of distinct dyes is recorded for quantitation (Timms, 2008). The quantitative differences obtained from the above mentioned studies are the first scan of the differentiators, which are the storehouse of potential biomarkers and therapeutic targets. .

Selection of appropriate proteomic technology

Advocates of gel free and gel-based proteomics favour the method of choice due to certain advantages. Shot-gun proteomics carried out using LC-MS platforms are less laborious, more reproducible and capable of generating a larger profile (Wilkins, 2009). On the other hand, gel-based proteomics (Rogowska-Wrzesinska, 2013) aids identification and sequencing of proteins from organisms with minimal genomic information, efficiently identifies protein isoforms and proteins modified by glycosylation, proteolytic cleavage, etc.

Selection of comparison groups for identification of therapeutic targets

Identification of differentiators has been carried out with different aims and therefore differentiators are derived from diverse comparison groups. However, they inadvertently point at the key

molecules as potential therapeutic targets.

Comparison of tumor and normal in retrospective or prospective studies

For understanding cancer biology, the profile of differentiators are generated to reveal molecular mechanisms responsible for disease progression. Potential markers for progression of pulmonary squamous cell carcinoma were identified by examining samples of lung SCC and adjacent normal tissues using 2D-DIGE (Lihong, 2014). Markers of progression of oral squamous cell carcinoma from premaliganant lesion to carcinoma have been similarly demonstrated (Wang, 2009). Using gel free approach, insight into the underlying mechanisms of formation of polyploidy giant cancer cells (PGCC) and the relationship between PGCCs and cancer stem cells in patients with ovarian cancers has been established (Zhang, 2013).

Differential molecular profile is often generated for better stratification of tumors in order to improve diagnosis and management of the disease. Diagnostic markers have been identified by 2D-DIGE for cervical cancer (Canales, 2014; Guo, 2014). Similarly, using gel-based approach it has been shown that high expression level of Galectin-1 may correlate with development of nasopharyngeal carcinoma (NPC), and Galectin-1 as a potential diagnostic marker or therapeutic target for NPC (Tang, 2010). Further, a significant proportion of primary breast cancers are negative for estrogen receptors (ER), progesterone receptor (PgR), and Her2, comprising the triple negative breast cancer (TNBC) group. Women with TNBC have poor prognosis because of the aggressive nature of the tumors and current lack of suitable targeted therapies. The increased expression of Mage-A4 in the tumors enabled the detection of the protein in the tumor interstitial fluids and in sera. Immunotherapeutics approaches specifically targeted Mage-A4 protein, or Mage-A protein family members represents novel management options for TNBC (Cabezon, 2013).

An alternative aim for profiling of tumors is to predict prognosis. Using gel based proteomics approach, a subgroup of breast tumors with overexpressed C7 or f24 showed poor clinical outcome (Gromov, 2010). Similarly, LC-MS approach identified RBBP6 as prognostic marker for gastric cancer stem cell (Morisaki, 2014) and WD repeat containing protein 1 was identified as a diagnostic marker in the interstitial fluid from ovarian cancer (Haslene-Hox, 2013).

Comparison groups with focus on therapeutics

Several differentiators identified earlier may be useful potential therapeutic targets. Additionally, proteomic analyses aimed at understanding mechanism of drug resistance or drug action have greater probability to identify as therapeutic

targets.

Analysis of cell lines or samples from patients, untreated or treated with a drug, provides insight into the molecular mechanism of action of drugs/ chemo-preventive agents. Further identification of the drug modulated pathway may indicate therapeutic targets for further exploration. Curcumin, a natural anticancer agent, inhibits cell growth in a number of tumor cell lines and animal models. Molecular mechanism of curcumin induced apoptosis in different gastric cancer cell lines was studied by 2DGE (Cai, 2013). Similarly, using gel free approach, *Bifidobacterium* infantis thymidine kinase/ nucleoside analogue ganciclovir (BI-TK/ GCV) exhibited sustainable anti-tumor growth activity and induced apoptosis in bladder cancer, via peroxiredoxin I and NF kB pathway (Jiang, 2014).

Comparison of chemo-sensitive and -resistant tumors/cell lines provides information of the molecular basis of resistance and hence molecules to be considered as alternate therapeutic targets. In mantle cell lymphoma, a rare aggressive type of B cell non-Hodgkin's lymphoma, wherein response to chemotherapy tends to be short and patients relapse, the tumor necrosis factor related apoptosis inducing ligand (TRAIL) is a novel molecule with antitumor effects. In TRAIL resistant cases, 2DGE analysis demonstrated downregulation of the key enzymes of purine metabolism with profound effects on nucleotide homeostasis and can render cells vulnerable to further disruption of purine nucleotide metabolism. Thus proteins in this e pathway identifiedare potential therapeutic targets for selective elimination of resistant cells (Pospisilova, 2013). Chemo-resistance hinders effective treatment in several human cancers. HSP27 is as an alternate target for anticancer drug developmentin gemcitabine therapy resistant pancreatic cancer (Liu, 2012). Histone deacetylase inhibitors (HDACi) demonstrates anticancer activities and used in combination therapy. In lymphoid cell lines, 2DGE analysis has identified HSPA1A as an overexpression with resistance to valproic acid HDAC inhibitor. *In vitro* experiments demonstrate that treatment with KNK-437, an inhibitor of HSPA1A, enhanced cytotoxic effects of valproic acid, thereby identifying HSPA1A as a possible therapeutic target, in combination with HDACi, for lymphoid neoplasms (Fuji, 2012).

Authentication of the potential of identified therapeutic target

Differentiators have been identified for several cancers, however, differentiators as a therapeutic targets needs further investigations. Bioinformatic tools for pathway identification are extensively used to find a functional link between the differentiators. A molecule in a pathway associated with hallmarks of cancer

(Hanahan, 2011), qualify as potential therapeutic targets. However, the potential needs to be authenticated experimentally. In several studies, over expression or activation as well as down-regulation or inhibition of the identified potential drug target is used to demonstrate the effect on tumor promotion or progression.

Tamoxifen (Tam) is a widely used selective estrogen receptor modulator (SERM) for treatment of hormone-responsive breast cancer and acts via inhibition of E6AP expression identified as a differentiator by 2DGE. Authentication of E6AP as a therapeutic target was achieved by demonstration of Tam- and siE6AP-mediated inhibition of E6AP with subsequent enhanced G0-G1 growth arrest and apoptosis (Lochab, 2012). Small interfering RNA (siRNA)-mediated knockdown confirmed a functional role for MDA-9 and GRP78 in promoting cell invasion in A375 cells (Guan, 2014). Similarly in liposarcoma, an aggressive cancer with poor outcome, gankyrin oncoprotein showed a significantly high expression. Inhibition of gankyrin led to reduction of *in vitro* cell proliferation, colony-formation and migration, besides *in vivo* dedifferentiated liposarcoma tumorigenesis (Hwang, 2014). KvLQT1 channel blockade was showed efficient reduction of A549 and H460 cell proliferation and migration. Moreover, KvLQT1 overexpression in AD samples suggested it to be a potential therapeutic target in lung cancer (Girault, 2014). In an *ex vivo* model, siRNA mediated inhibition of HSP70, showed dose-dependent inhibition of cell growth and burst formation unit erythroid (BFU-E), increased apoptosis in the erythroid lineage and decreased pJAK2 signaling. Thus HSP70 as a potential therapeutic target in myeloproliferative neoplasms especially polycythemia vera was confirmed (Gallardo, 2013). Similarly inhibition of Apg-2 showed decreased cell proliferation and induced apoptosis in BCR/ABL positive cells, indicating an additional therapeutic target for chronic myeloid leukemia (Li, 2013). Whereas, in chronic lymphocytic leukemia (CLL) a clonal malignancy with immense clinical heterogeneity with variable prognosis, hyper reactivity of the B cell receptor (BCR) to unknown antigen ligation plays a pivotal role in CLL survival. Proteomic analysis revealed that kininogen, a critical protein of kinin-kallikrein system, was upregulated upon BCR stimulation and mayprovide a therapeutic target in CLL (Kashuba, 2013). Further, in MLL-rearranged leukemia, TET1 was identified as a potential therapeutic target (Huang, 2013). Similarly in endometrial cancer, overexpression of bone marrow stromal antigen 2 (BST2) was detected in LC-MS analysis and confirmed by immuno-histochemistry using clinical samples. In an *in vivo* xenograft model, BST2 antibody treatment inhibited tumor growth of BST2-positive endometrial cancer cells in an NK cell-dependent manner,

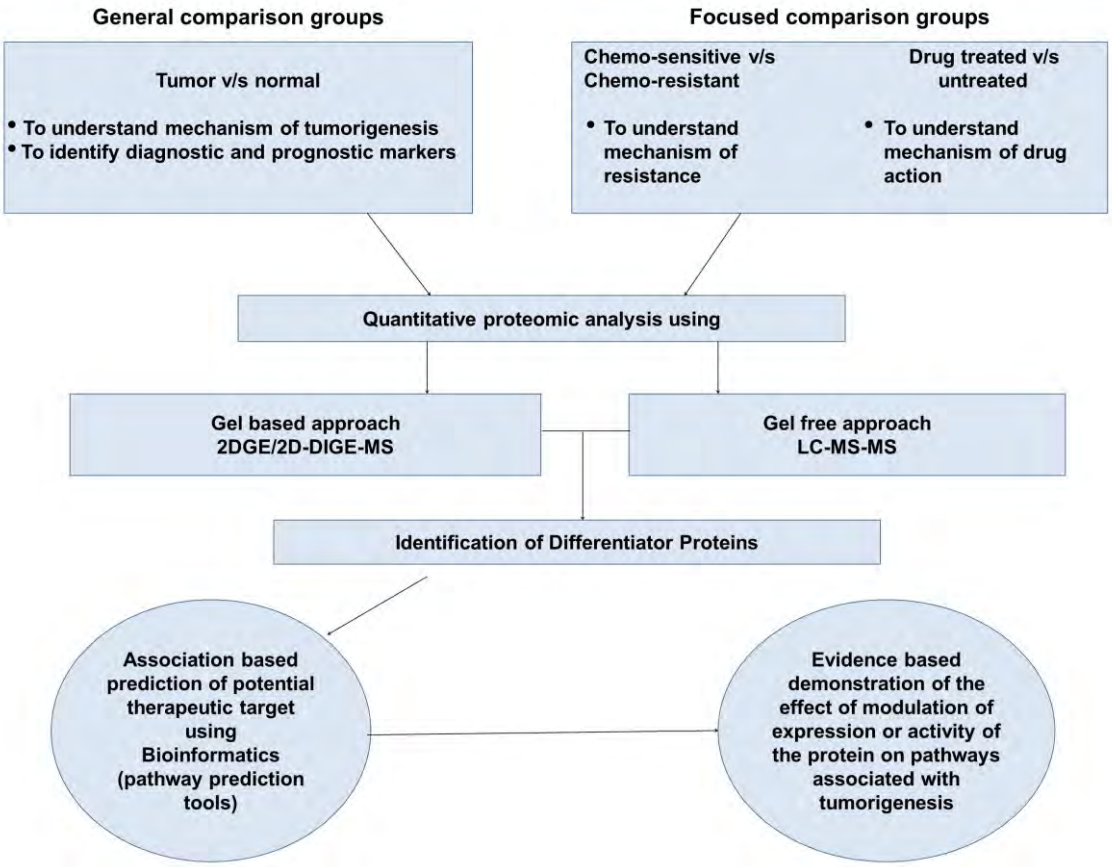

Figure 1: Various strategies and technologies for proteomic identification of therapeutic targets for cancer. (2D-DIGE: two dimensional difference gel electrophoresis; MS- mass spectrometry).

advocating candidacy of BST2 as a therapeutic target (Yokoyama, 2013). These studies provide an initial authentication of the potential of identified therapeutic targets.

To summarize, the review highlights usefulness of proteomic technology in identification of therapeutic targets as outlined in figure 1. The review reveals that differentiators, identified by both gel-based and gel free approaches qualify therapeutic targets. It appears that comparative proteomic analysis of chemosensitive and chemoresistant cells as well as that of drug treated and untreated cells, are useful in identification of therapeutic targets. The search strategy for therapeutic targets has evolved from association based approaches wherein a differentiator protein with known role in key functional pathways qualified as a potential target. Evidence based selection of therapeutic targets necessitated experimental demonstration of the ability of the differentiator to affect the hallmarks of cancer (Guo, 2013). Thus, we conclude that advances in proteomic technology and refinements in experimental strategies have contributed to identification of therapeutic targets in tumors and in turn to the field of targeted therapeutics.

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