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Minireview

Challenges in cancer research and multifaceted approaches for cancer biomarker quest

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ABSTRACT

Recent advances in cancer biology have subsequently led to the development of new molecularly targeted anti-cancer agents that can effectively hit cancer-related proteins and pathways. Despite better insight into genomic aberrations and diversity of cancer phenotypes, it is apparent that proteomics too deserves attention in cancer research. Currently, a wide range of proteomic technologies are being used in quest for new cancer biomarkers with effective use. These, together with newer technologies such as multiplex assays could significantly contribute to the discovery and development of selective and specific cancer biomarkers with diagnostic or prognostic values for monitoring the disease state. This review attempts to illustrate recent advances in the field of cancer biomarkers and multifaceted approaches undertaken in combating cancer.

1. Cancer biology

Cancers represent group of unprecedentedly heterogeneous diseases that affect humans with high frequency and contribute significantly to overall morbidity and mortality. Currently there are only limited anti-cancer therapies available with highly variable efficacy, which reflects an unsatisfactory cure rates. The on-set of carcinogenesis is usually initiated with DNA alteration in the target cell that mostly arise as a result of combination of several factors including genetics, environmental, dietary, immune and as well as several others still to be discovered. The DNA alterations may include a single point nucleotide exchange, deletion, amplification, translocation, chromosomal rearrangement, methylation or other events that can then lead to the activation of oncogenes and inactivation of tumor suppressor genes. Usually, an accumulation of DNA alterations irreversibly transforms normal cell into dysplastic and cancerous one. Previously, it was assumed that dysregulation of limited amount of oncogenes or tumor-suppressor genes caused cancers, however, more recently the alterations of many other genes directly or indirectly involved in the carcinogenesis and tumor progression process have been demonstrated. The integration of known cancer genes into protein transduction and signalling networks reveals their characteristic interactions within networks and shows that cancer genes often function as 'network hub proteins' which are involved in many cellular processes and form focal nodes mediating cross-talk between different signalling pathways [1–3]. Hence such disruptions in cellular communication significantly contribute to cancer development and progression. The

Abbreviations: MAP, mitogen-activated protein; PI3K, phosphatidylinositol-3 kinase; mTOR, mammalian target of rapamycin; Prom1, Prominin 1; EGFRs, epidermal growth factor receptors; VEGFR, vascular endothelial growth factor receptor; PDGFRs, platelet-derived growth factor receptors; SCF, stem cell factor; Erk, extracellular-regulated kinase; Mek, MAP Erk kinase; MAPK, MAP kinase; PKB, protein kinase B; Her-2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; DASL, cDNA-mediated annealing, selection, extension, and ligation; FFPE, formalin fixed paraffin-embedded; miRNAs, microRNAs; 1-D and 2-D gel electrophoresis, one- and two-dimensional gel electrophoresis; 2-D HPLC, two-dimensional high pressure liquid chromatography; DIGE, difference in gel electrophoresis; MS, mass spectrometry; MALDI, matrix assisted laser desorption ionisation; TOF, time-of-flight; SELDI, surface enhanced laser desorption ionisation; SILAC, stable isotope labeling by amino acids in cell culture; ICPL, isotope-coded protein labels; iTRAQ, isobaric tags for relative and absolute quantification; AQUA, absolute quantification; MRM, multiple reaction monitoring; FT-ICR, Fourier transformation-ion cyclotron resonance; GIST, gastrointestinal stromal tumor; MudPIT, multidimensional protein identification technology; NCI-60, the US National Cancer Institute 60 human tumor cell line anti-cancer drug screen; SSRP1, structure specific recognition protein 1; RALBP1, Ral binding protein 1; HSP27, heat shock 27 kDa protein; CDKI, cyclin-dependent kinase inhibitor

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molecular alterations are frequently found in processes mediated by growth factors, hormones and cytokines via their receptors. In order to understand cancers better, the roles of tyrosine kinase receptors and signal transduction pathways such as the epidermal growth factor receptors, platelet-derived growth factor receptors, vascular endothelial growth factor receptors, the Ras/Raf/mitogen-activated protein (MAP)-kinase, phosphatidylinositol-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and other pathways have been studied and therapeutically targeted in different cancers [4–6]. In 2000 Hanahan and Weinberg [7] outlined the basic principles of a malignant process and proposed that human cancers should demonstrate a minimal set of capabilities that are necessary to progress to a malignant tumor state: (1) growing uncontrollably; (2) insensitivity to anti-growth signals; (3) evading apoptosis; (4) acquiring limitless replicative potential; (5) ability to form and sustain new blood vessels, e.g. angiogenesis; and (6) invasion and metastasis. The identification of altered genes and signalling pathways and their targets could lead to substantial improvement in prevention, diagnosis, prognosis and tailored therapy of cancers.

Recent studies have revealed that apart from the genetic abnormality of many cancer-related genes/proteins, epigenetic regulation of genes is also critically important in the process of carcinogenesis. The mechanisms of epigenetic control of genes involve changes of gene expression patterns mediated by modifications of DNA and/or histones, without the direct alteration of nucleotide sequence of the genes. Those modifications include several processes, mainly the DNA methylation and the covalent modifications such as acetylation, methylation, phosphorylation and ubiquitination of specific amino acid residues of the N-termini of the core histones [8]. Among these modifications, histone acetylation/deacetylation plays a central role in epigenetic regulation of gene expression. Typically, high acetylation level of the chromatin hallmarks the active transcription of the genes, whereas transcriptionally inactive chromatin is usually characterised by low acetylation level of histones. It has been discovered that the occurrence of many cancers is accompanied by a genome-wide histone hypoacetylation and indeed, histone deacetylase inhibitors have significant and clinically proven anti-tumor activity [9,10].

The other key question in current cancer biology is the origin and role of cancer stem cells. Cancer stem cells are remarkably similar to normal stem cells in respect to their ability to self-renew and be multipotent [11–13], but until now it was not clear whether cancer stem cells are the direct progeny of mutated stem cells or more mature cells that re-acquire stem cell characteristics during tumor formation. Using an inducible Cre, nuclear LacZ reporter allele knocked into the Prominin1 locus (Prom1(C-L), Zhu et al. [14] showed that Prom1(+) enterocyte precursor cells located at the base of crypts in the small intestine represent small intestinal stem cell. Furthermore, lineage-tracing studies of adult Prom1(+/C-L) mice after activation of endogenous Wnt signalling pathway demonstrated disruption of crypt architecture with neoplastic transformation and importantly only progeny of Prom1(+) cells retaining Prom1 dependent reporter gene expression marked the fraction of cells that are susceptible to neoplastic transformation. Stem cells are typically expressing the multi-drug resistance proteins and transporters [15]. Indeed, the cancer stem cells are capable of resisting chemotherapy and/or radiotherapy, and they may be responsible for failure of anti-cancer treatments. Hence, the understanding of cancer stem cells properties could help to eliminate them and to prevent re-growth of cancer [16,17].

2. Anti-cancer therapies

Most of currently used conventional anti-cancer treatments include surgery, radiotherapy and chemotherapy. Unlike first two local and/or loco regional therapeutic modalities, chemotherapy has systemic effects and it can eliminate disseminated tumor cells, which are the ultimate reason for tumor recurrence and treatment failure even in potentially curable stages. The chemotherapy in oncology is focused on application of cytostatic and/or cytotoxic drugs with potential to kill malignant cells. Such drugs may be obtained using chemical synthesis or isolated and/or derivatised from plants or different fungi and such cytotoxic and cytostatic drugs are known to frequently induce DNA damage and/or block cellular division. These effects are mediated via a broad spectrum of mechanisms and these in turn target both the tumor and rapidly proliferating normal tissues. Indeed, dose of anti-cancer drugs is a balancing act between achieving concentrations that are effective towards the malignancy and that result in acceptable side effects. The differences in proliferation rates, expression of specific targets and multi-drug resistance proteins between cancer and healthy cells together with various concentrations and metabolism of individual cytostatic drugs in body organs or tissues are major determinants of drug effects. In spite of all precautionary efforts, frequency of secondary side effects and toxicity remains high. Neutropenia is one common example of a side effect of many anti-cancer agents and is related to an increased risk of infection and mortality [18]. This also reflects the general situation that therapeutic standards are based on average efficacy determined by controlled clinical trials involving large cohort of patients and generally extended to the whole population. However, for many patients, the gold standard treatment translates into very low response rate, individual efficacy is far from the mean and this becomes evident only a few months after drug administration. Currently approved anti-cancer drugs are sorted into several groups according to known mechanisms inducing DNA damage and cellular death and include: (i) alkylating cytostatic agents (melphalan, chlorambucil, cyclophosphamide and ifosfamide, derivatives of nitrosourea – carmustine and lomustine, busulfan, dacarbazine, temozolomide and procarbazine); (ii) anti-metabolites (methotrexate, 5-fluorouracil and its derivative capecitabine, cytosinarabinoside, gemcitabine, mercaptopurine, fludarabine, cladribine, hydroxyurea); (iii) anti-cancer antibiotics (doxorubicine, idarubicine, epirubicine, mitoxantron, bleomycine, mitomycin C); (iv) plant alkaloids (vinca alkaloids – vincristine, vinblastine and vinorelbine, podofylotoxin alkaloids – etoposide and teniposide, camptothecin analogs – topotecan and irinotecan, taxanes – paclitaxel and docetaxel); and (v) other drugs (platinum compounds – cisplatin, carboplatin, oxaliplatin; L-asparaginase; amsacrin and others).

Recent advances in our understanding of cancer biology including cancer-related genes, proteins and signalling pathways have opened up a window for tailored and personalised anti-cancer therapy. The ultimate goal would be tailored drug therapies using active dosage firmly directed at specific tumors with a defined molecular profile. Molecular processes in cancer cells include specific alterations in signalling pathways and protein interaction regulatory network such as over-expression and mutations of cellular receptors and other downstream effectors. Such emerging knowledge subsequently has led to the development of new molecular targeted anti-cancer agents that hit effectively cancer-related proteins and pathways. Several approaches have been applied including inhibition of growth factor receptors and receptor tyrosine kinases (EGFRs, VEGFR, PDGFRs, stem cell factor (SCF), c-Kit and chimeric Bcr-Abl protein) [19–21], inhibition of intracellular effectors that mediate intracellular oncogenic signaling such as Ras-Raf- Mek-Erk MAPK or AKT (PKB) and mTOR downstream effectors of the PI3K pathway [22], and other agents targeting integrins involved in angiogenesis and invasion, selective inhibitors of histone deacetylases, ubiquitin–proteasome system and matrix metalloproteinases [5,23].

Although the most of the molecular targeted agents have presented cytostatic activity with favorable degree of cytotoxicity as monotherapies, only modest effects or failure in survival benefit have been observed in advanced tumors. Therefore, several strategies using combination therapy have been suggested to explore possible combination of two or more agents that may target the same pathway more effectively or two different pathways, where the additional one, such as angiogenesis, may complement original target to increase treatment efficacy. Other possible therapeutic strategies include the use of molecular multi-target agents or multi-modality treatment like a combination of molecularly targeted agents with conventional chemotherapy [24–28].

3. Impact of genomics and transcriptomics on classification of cancers and prediction of response to anti-cancer therapy

Today, many cancers are diagnosed and confirmed by pathologists. Anatomic staging plays a significant role in making a diagnosis and treatment decision and classical pathological indexes such as grading are used to make prognosis and predict development of metastatic disease or help guide selection of a primary therapy. However, results of recent studies have shown that the use of these morphology-based tools is not sufficient enough in accurate identification and classification of many different disease subtypes in cancer patients. Several approaches are now available to detect genomic changes leading to tumor genesis such as fluorescence in situ hybridisation, comparative genomic hybridisation and single nucleotide polymorphism analysis including samples isolated by laser-capture micro-dissection. Furthermore, integrating genomic and transcriptomic data may significantly enhance characterisation of molecular fingerprints associated with specific cancer phenotypes [29–31]. Transcriptomic studies are focused on changes in mRNA abundance and alterations in alternative splicing [32]. Analysis of mRNA expression using cDNA microarray permits high throughput gene expression profiling and represents a powerful screening technique allowing analysis of the activation state of cancer-related genes and characterisation of molecular signatures differentially expressed or spliced genes between normal and cancer tissue. This molecular approach has allowed the subdivision and classification of cancers into more homogeneous subgroups with specific features [29,33–36]. For example Sorlie et al. [37] identified set of 456 genes that could classify breast tumor into molecular subtypes (luminal type A and B, Her-2/neu positive, basal-like and normal breast-like subtype) and validated these results on independent cohort of patients with locally advanced breast cancer. They also showed that those molecular subtypes can be clinically distinguished based on estrogen/progesterone receptor (ER/PR) and Her-2/neu gene positivity: ER/PR positive luminal subtypes A and B; ER+PR negative but Her-2/neu positive; ER-PR-Her-2/neu-triple negative basal-like; and human epidermal growth factor receptor 2 (Her-2) negative normal breast-like cancers. However, this study also provided additional molecular features that did not reflect clinical criteria such as lymph node stage, age and menopausal status of patients, thus underlying significant contribution of molecular transcriptional profiling in objective classification of cancers.

Despite the usefulness of microarray data in characterisation of tumor subtypes, recent studies have focused on monitoring of anti-cancer therapy and prediction of tumor response. The work has been done to ascertain expression patterns for commonly used chemotherapy like antimetabolite 5-fluorouracil [38], mitotic poison docetaxel [39], estrogen receptor antagonist tamoxifen [40] and chemotherapy regimens using combined fluorouracil, doxorubicine and cyclophosphamide in neoadjuvant settings [41]. More recently, this type of studies have been also extended to prediction of therapeutic outcome in targeted anti-cancer therapies [42]. The microarray analysis has also been a valuable tool for demonstrating the importance of tumor milieu. Using this technology, it has been possible to demonstrate significant changes in expression of cancer-related genes in cancer cells grown in a 3-D matrix as compared to 2-D culture when treated with anti-cancer drugs and compared to control untreated cells. Findings from such studies have highlighted the importance of cellular architecture, phenotypic variations, and extracellular matrix barrier to drug transport in cellular response to selected compounds. Combination of all these parameters contributes to the overall drug efficacy for that particular cancer or tumor [43]. These studies are aimed at the discovery of multigenic biomarkers with higher probability to better describe complex biology systems. Disease and/or therapy specific transcriptional profiles are followed by extensive validations in order to demonstrate clinical relevance. Whilst microarray technologies are extremely useful and hold their place in research and possibly in diagnostic arenas, there are still some associated limitations that restrict their full use in the clinical environment. Once these have been resolved, microarray technology will be at the forefront of clinical and scientific research. Besides the obvious technical problems associated with platform standardisation (reference RNA and housekeeping genes), reproducibility (printing fluctuation, spot variations, gene oligonucleotide tags and processing), image analysis data processing (need for new bioinformatics and biostatistical tools), there are two major caveats – essential requirement for freshly-frozen tumor tissue or cells and lack of knowledge on specific functions of many genes [34]. Inconvenient sample collection, processing and storage of frozen samples for microarray studies restrict their use for relatively small sample sets. Additionally, the resulting classifier genes require extensive validation by other techniques including qRT-PCR or DASL (cDNA mediated annealing, selection, extension and ligation). Indisputable advantage of qRT-PCR or DASL is that they can be performed using formalin fixed paraffin-embedded (FFPE) tumor tissue samples that present priceless source of clinical samples for investigation. The qRT-PCR utilises amplicons which are specifically designed on DNA fragments usually less than 100 bp thus providing high efficiency and making it suitable for examination of gene expression variation in fixed samples containing chemically modified and fragmented RNA. Compared to microarray analysis, qRT-PCR is amenable to analysis of a high number of samples in retrospective studies. The DASL assay has been developed to combine the advantages of microarray and qRT-PCR technologies. Assay allows sensitive and reproducible gene expression profiling for parallel analysis of several hundreds of mRNA transcripts on the BeadArray platform. Since this assay can be performed on FFPE samples, this technology may be especially useful for determining cancer prognosis or therapy response taking advantages of retrospective investigations of samples with known clinical outcome [44–46].

Recent work has revealed a class of microRNAs (miRNAs), a small non-protein coding RNAs which function as negative gene regulators at the post-transcriptional level. miRNAs regulate multiple gene targets and are capable of controlling wide range of biological processes including growth, differentiation and apoptosis. Hence, disregulation of miRNAs may contribute to many diseases that are known to arise from imbalance in regulation of these cellular processes. It has been shown that mutations and disruption in miRNAs biogenesis are also involved in cancer [47,48]. Studies by Lu et al. reported express profiling analysis of 217 mammalian miRNAs in 334 samples of a variety of human cancers. Expression miRNAs profiles grouped tumors according to tissue of origin and their comparison in tumor versus normal tissue reflected the degree of cell differentiation [49]. The authors introduced a novel bead-based flow cytometric miRNA profiling method taking advantage of Luminex xMAP technology where up to 100 distinct

sets of color-codes beads can be utilised. Each bead set is coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Within the Luminex compact analyser, lasers excite the internal dyes that identify each microsphere particle and also any reporter dye captured during the assay. Many readings are made on each bead set, further validating the results. This method applied to miRNA, couples oligonucleotides that are complementary to appropriate miRNAs onto beads. Purified miRNAs from cell or tissue samples are hybridised to the oligo-bound beads. Bead-based hybridisation has the theoretical advantage that it might more closely approximate hybridisation in solution, and as such, the specificity could be expected to be superior to chip microarray hybridisation. This multiplex method also set the stage for future protein/nucleic acid co-detection (http://www.luminexcorp.com/technology/index.html). Hence this technology together with novel antibodies and various specific biomarkers panel such as cancers, cellular signalling, cytokines, chemokines, growth factors, endocrines and metabolic markers, represents a powerful complete solution for 'in-depth' studies on various cancers and other diseases.

4. Proteomics in cancer research

Despite better understanding of genomic aberrations of cancer cells and advances in characterising diversity of cancer phenotypes via transcriptomics, it is obvious that proteomics deserves attention in cancer research. Compared to genomic and transcriptomic classifiers, proteomic features appear to be more realistic platforms for identification of cancer-related alterations in molecules and signalling pathways and could therefore significantly contribute to our understanding of cancerogenic developments. There are several major reasons why one should focus on cancer proteomes: (i) there is generally a poor correlation between transcriptional levels of many genes and relative abundances of corresponding proteins; (ii) due to differential splicing and translation each gene may encode several different protein variants with different properties; (iii) the key proteins driving malignant behavior of cancer cells can undergo post-translational modifications including phosphorylation and glycosylation; and (iv) proteomes are capable of monitoring dynamic changes, hence they can be used to follow up the course of the disease and any response to drug therapy. However, comprehensive studies on cancer proteomes present an important challenge and involvement of (i) extreme heterogeneity of tissue cell populations which are part of the cancer itself or host response to developing malignancy; (ii) broad dynamic range of protein abundances in proximal body fluids and serum/blood plasma which present a rich source for biomarker identification; and (iii) the need for validation of molecular changes in independent cohort of cancer patients using independent techniques and functional assays. With these aspects in mind, applications of proteomics to cancer research require careful consideration of currently available or emerging novel proteomic technologies in order to provide results that can be relevant for biological and functional interpretation. More importantly, to move forward in the fight against cancers, the proteomic findings need to be successfully translated into clinical oncology [50].

A variety of approaches that can be utilised in cancer proteomics require proper sample preparation including sample-enrichment strategies focused on subcellular or organelle fractionations, phosphoproteins and glycoproteins, specific protein tagging or tissue micro-dissection. Following proteomic analyses of cancer tissues, cells and/or body fluids are subjected to global profiling and/or comparative functional analysis. Protein separation-based approaches most frequently explore gel-based techniques (1-D and 2-D gel electrophoresis), however, gel-free liquid phase chromatographic approaches (2-D HPLC) offer some advantages and provide results complementary to gel-based fractionation [51]. The major bottleneck of classical 2-DE based approach suffers from very low dynamic range of quantification using various staining procedures and only moderate reproducibility. The use of fluorescence protein labelling and running the mixture of two different samples in one gel known as difference in gel electrophoresis (DIGE) technique has provided researchers with improved tool to look closer at the biomarkers of interest but did not completely solve the problems associated with gel-based techniques as well as protein sample complexity or high dynamic range of protein abundances in contrast to rather low dynamic range of protein detection and quantification [52].

Mass spectrometry (MS) now permits the identification of many proteins with high sensitivity that is mostly dependent on ionisation efficiency of a given peptide. Sensitivity is high for purified peptides whilst it decreases with the complexity of the samples. Thus, the quality of protein fractionation has high impact on protein identification coverage – in complex protein mixtures it is difficult to detect low abundant peptides/proteins in the presence of highly abundant ones. This situation is typical for many body fluids and tissues as well as detection of post-translationally modified peptides in the presence of unmodified counterparts. At this stage, mass spectrometry is not quantitative but allows relative comparison between same peptides/proteins in a set of different samples. MALDI-TOF (matrix assisted laser desorption ionisation-time-of-flight) is now routinely used for the identification of proteins. The SELDI (surface enhanced laser desorption ionisation) with TOF mass spectrometer combines effective sample prefractionation based on capturing different types of proteins such as hydrophobic, hydrophilic, ionic, ligands, antibodies and others on selective types of surfaces with mass analysis. This chip-based procedure is a relatively straightforward technology that is easily applicable in the clinical environment. The limitation has been the low mass accuracy which cannot be used for assignment of protein/peptide identifications.

Hence, a need for newer technologies was highlighted for progress in the field of cancer biomarker discovery. Modern mass spectrometry-based quantification methods have gained popularity over the last 5 years. The determination of protein abundances in samples is performed using stable isotope labelling techniques including stable isotope labelling by amino acids in cell culture (SILAC) or isotope-coded protein labels (ICPL) prior protein fractionation. In addition, an improved approach using amine-reactive isobaric tags for relative and absolute quantitation (iTRAQ) has been developed with potential usefulness. This technique is based on chemically tagging the N-terminus of peptides generated from protein digests that have been isolated for instance from cells cultured under different experimental conditions or other biological materials [53,54]. A specific way to introduce isotope label into peptide is the use of trypsin-catalysed incorporation of ^{18}O during protein digestion. The incorporated labels create specific mass tags that can be recognised by mass spectrometry and at the same time provide the basis for quantification. Unlike relative quantification, absolute quantification (AQUA) can be achieved using internal standard by adding known quantity of stable isotope-labeled standard proteotypic peptide to a protein digest and subsequent comparison of mass spectrometric signals of standard labeled peptide and corresponding endogenous peptide in digest. The specific modification of AQUA is a method called multiple reaction monitoring (MRM) in which the mass spectrometer monitors not only intact peptide mass but also one or more specific fragmentations of those peptides. The combination of retention time, peptide mass and fragment mass eliminates ambiguities in peptide assignment and allows for higher range of quantification. Also, targeted fragmentation of selected ions allows parallel quantification of hundreds of candidate genes in the sample in very short period of

time. Development of hybrid methods coupling MRM assay with protein enrichment by immuno-depletion or enrichment of the peptides by antibody capture and further improvements may be capable of extending MRM method to full dynamic range of plasma proteins. With this view in mind, MRM measurement of plasma peptide may provide a rapid and specific assay platform for high throughput biomarker validation [55,56].

Due to problems posed by isotope labelling methods such as removal of contaminating reagents, sample loss, costs and reproducibility, label-free methodologies for quantitation were introduced. One of the label-free method is the protein microarray methodology. Each array can contain hundreds or thousands of immobilised proteins or antibodies, where the specificity of the protein or antibody, respectively, is crucial [57]. Microarrays are versatile tools for examining large number of samples for biomarkers studies in various environments such as drug development and clinical trials. Protein and tissue microarray technologies provide a high-throughput platform for protein expression investigations including tissue sections, thus allowing analysis of several hundreds of samples simultaneously (Fig. 1). Moreover, the advent of digital slide technology has afforded an unparalleled opportunity for archiving valuable histopathological and immunohistochemical specimens. Such digital images offer a unique opportunity to provide a paradigm shift in relation to interpretation of such data via the use of automated image analysis algorithms [58,59].

Currently, proteomic analyses have been extended to whole tissue sections by using MALDI imaging. This approach allows detection of proteins in situ in the tissue sections and analysis of their spatial distribution. Each MALDI imaging data set containing large number of mass spectra may be evaluated using hierarchical clustering that is helpful in classification and interpretation of complex human tissue [60].

Recently, direct tissue proteomic approach, which represents modified shotgun proteomic tool enabling identification of proteins by micro-reverse phase LC–MS/MS isolated from FFPE tissue was introduced [61]. The successful application of direct tissue proteomics needs improved extraction protocols from FFPE tissue samples, but it offers retrospective investigation of biomarkers in well characterised clinical samples. The combination of this technology with laser-capture micro-dissection is expected to facilitate mapping of global signalling networks, which would be a powerful and more informative than individual markers in diagnostics, disease prognosis and tailored cancer therapy.

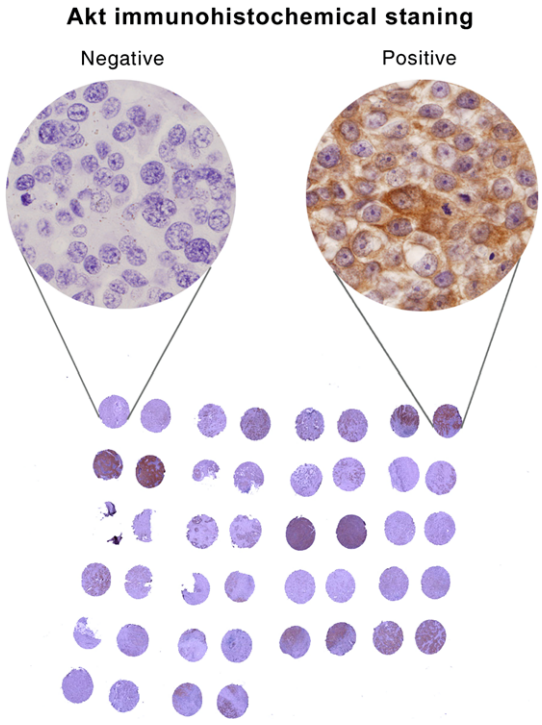

Fig. 1. Biomarker validation in tissue microarrays, example of pan anti-Akt kinase staining using primary rabbit polyclonal antibodies (11E7, Cell Signaling, USA).

5. Contribution to cancer biomarkers development

The application of a wide range of proteomic methods to the discovery of new cancer protein biomarkers would help to identify early markers of disease as well as help with prognosis, prediction and monitoring of patient response to a particular therapeutic intervention (Table 1). Such markers of disease could be released directly by cancer cells or may represent a part of host's response to malignancy. These types of diagnostic candidates could be expected to be detected in body fluids and hence detection is vitally important. The cellular proteins are often shed into extracellular fluids including tissue interstitial fluids and blood plasma. Whilst tissue interstitial fluids are in direct contact with tissue/cells via transfer of molecules, the composition of blood plasma results from the communication with tissue interstitial fluids. On the other hand, the blood plasma influences the composition of other body fluids. It is important to realise that relative concentration of biomarkers is highest in tissue interstitial fluids, which in turn drain into lymph and lymph vessels from different tissues finally merging and draining into the blood. As a result, the final concentration of biomarkers in blood would be significantly lower compared to interstitial fluids. Hence, various body fluids represent more or less enriched source of biomarkers [62]. Application of SELDI-TOF in the analysis of serum in a variety of cancers has been most frequently used [63]. In order to develop a new workflow for biomarker candidate identification, Lopez et al. [64] evaluated high-throughput carrier protein-bound affinity enrichment of serum samples coupled with high-resolution MALDI orthogonal TOF-MS, discriminant analysis of the resulting mass spectral patterns, and sequence identification of the discriminating ions to search for putative early protein/peptide biomarkers in ovarian cancer serum samples. This approach yielded a number of discriminating peptides, and provided three marker sets (9, 4, and 7 markers) that enabled classification of samples from cancer patients and healthy controls. Peptide fragments associated with the coagulation cascade provided the highest classification power. Among other candidates were identified lower intensity peptides corresponding to casein kinase 2, transgelin, keratin 2, glycosyl transferase (LARGE), and diamino oxidase. The sets of the discriminating carrier-protein bound fragments differentiated samples from patients with ovarian cancer and from apparently healthy controls with sensitivities and specificities of up to 93% and 97%, respectively. Using various proteomic approaches, the quest for cancer biomarkers in plasma is currently underway for particular cancers including breast, melanoma, lung, and pancreatic [65,66]. Among different proteomic technologies, serum glycomics approach is very attractive because the glycosylation of proteins is known to change in tumor cells during the development of cancer and these glycosylation changes correlate with increasing tumor burden and poor prognosis. Whilst currently used antibody-based immunochemical tests for cancer biomarkers of ovarian (CA125), breast (CA27.29 or CA15-3), pancreatic, gastric, and colonic (CA19-9) carcinomas target highly glycosylated mucin proteins, recent developments in MALDI-Fourier transformation-ion cyclotron resonance (FT-ICR) MS have allowed more detailed analysis of free glycan species resulting in glycan profiles containing distinct glycan features that may correspond to glycan "signatures of cancer" [67,68]. However, to further enhance the research progress it is essential to overcome limitations

Table 1
Proteomic technologies advancing cancer biomarker research.

Methodologies	Technology type with abbreviation	Research applications and usefulness	Cancer research area	Ref.
Gel-based methods	2-D-DIGE	Diagnostic biomarkers	Colorectal cancer	[87]
			Pancreatic adenocarcinoma	[88]
			Non-small-cell lung cancer	[89]
		Diagnostic and prognostic biomarkers	Bone and soft-tissue sarcoma	[90]
			Bladder cancer	[91]
		Screening of resistance biomarkers	Ovarian cancer	[92,93]
		Prognostic biomarkers	Hepatocellular carcinoma	[94]
			Lung adenocarcinoma	[95]
	2-D-PAGE	Diagnostic biomarkers	Papillary thyroid cancer	[96]
		Prognostic biomarkers	Gastrointestinal stromal tumor	[71]
Mass spectrometry-based methods – with isotope labeling	iTRAQ-2-D-LC-MS/MS	Prognostic biomarkers involved in metastatic processes	Breast cancer	[97]
		Diagnostic and prognostic biomarkers	Prostate cancer	[98]
		Diagnostic biomarkers	Head and neck spinocellular carcinoma	[99]
			Hepatocellular carcinoma	[100]
	ICAT-MS/MS	Diagnostic biomarkers in pancreatic juice	Pancreatic cancer	[101]
	SILAC-LTQ-FT-MS/MS	Diagnostic biomarkers	Hepatocellular carcinoma	[102]
	SILAC-LC-MS/MS	Diagnostic biomarkers	Pancreatic cancer	[103]
	^{18}O labeling MS	Diagnostic biomarkers	Cholangiocarcinoma	[104]
	$^{12}C6$- or $^{13}C6$-NBS labeling followed by MALDI-QIT-TOF	Diagnostic glycoprotein biomarkers in patient serum	Lung adenocarcinoma	[105]
Mass spectrometry-based methods - label-free	LC–MS/MS and MRM	Diagnostic biomarkers	Mouse model of breast cancer	[106]
	1-D-LC/MS/MS	Diagnostic biomarkers	Non-small-cell lung cancer	[107]
	LC–MS based	Prognostic biomarkers	FFPE archival melanoma tissues	[108]
	SELDI-TOF-MS	Diagnostic biomarkers in patient serum (mostly distinguish cancer patients from healthy individuals)	Laryngeal squamous cell carcinoma	[109]
			Lung cancer	[110]
			Gastric cancer	[111]
			Classifications of chronic hepatitis B, liver cirrhosis and hepatocellular carcinoma	[112]
			Renal cell carcinoma	[113]
		Diagnostic biomarkers in pancreatic juice	Malignant intraductal papillary mucinous neoplasm	[114]
		Diagnostic biomarkers in tissues	Renal cell carcinoma	[115]
		Biomarkers for prediction of clinical responsiveness to treatment	TRAIL and etoposide treated breast cancer and non-transformed cells	[116]
	MALDI imaging	Diagnostic biomarkers	Prostate cancer development	[117]
			Prostate cancer	[118]
Array-based methods	Antibody arrays	Diagnostic biomarkers	Esophageal adenocarcinoma	[119]
			Microarrays designed to detect circulating auto-antibodies against 51 tumor-associated antigens	
			Helicobacter pylori infection and gastric adenocarcinoma	[120]
			Ovarian cancer	[73]
		Diagnosis and prediction of therapy response	Bladder cancer	[121]
		Biomarkers monitoring therapeutic response	Her-2 (erbB-2) oncogene-dependent breast cancer	[122]
	Lectin glycoarrays	Diagnostic biomarkers	Colorectal cancer	[67]
	Tissue arrays	Prognostic significance	AQUA to analyze tumor-specific expression of vascular endothelial growth factor, VEGFR-1, VEGFR-2, and neuropilin-1 in breast cancer	[123]
			Use of combined biomarkers of oncogenic HPV and tumor suppressors of p53, pRb, and p21 in advanced TSCC provides prognostic molecular classification.	[124]
			E2-inducible histone variant H2A.Z, is significantly associated with lymph node metastasis and decreased breast cancer survival	[125]
Fluorescent-coded bead-based methods	Luminex®xMAP® technology	Diagnostic applications in clinical labs. Technology allows multi-analyte profiling with the ability to build internal controls into the various assays and offers multi-format and multiplexing capabilities for identifying either genetic or protein-based markers	Search for cancer markers in various cancers, offering great sensitivity to traditional ELISA-based systems, but with additional advantages including extended dynamic range and smaller sample size	[126]

Above are some examples of very recent studies utilising various proteomic technologies. More studies are recorded on Pubmed database – www.ncbi.nlm.nih.gov/pubmed/).

Table 2
Examples of most recent studies focused on response to anti-cancer treatment.

Anti-cancer agents	Methods	Cell lines	Up-regulated proteins	Down-regulated proteins	Ref.
Alkaloids					
Etoposide + TRAIL	SELDI-TOF-MS	MDA-MB231, ZR-75-1 and MCF-10A		Protein S100A6, ubiquitin	[116]
Vincristine	2-D-MALDI/TOF-MS	MCF-7	Heat shock 27 kDa protein	Heat shock 27 kDa protein	[80]
Vincristine	2-D-MALDI/TOF-MS	CEM	Class I β-tubulin, Actin (fragments), lamin B1	UV excision repair protein RAD23 homolog B, 40S ribosomal protein SA, heterogeneous nuclear ribonucleoprotein K, heterogeneous nuclear ribonucleoprotein F, p59 protein, ADP sugar pyrophosphatase, 14-3-3β, 14-3-3ε and 14-3-3τ protein, translationally-controlled tumor protein, L-plastin, lamin B1, 26S proteasome, α-tubulin 1, α-tubulin 4	[127]
Alkylating agents					
Temozolomid	2-D-LC/MS	U87-MG	Vimentin, RhoA	47-kDa mannose 6-phosphate receptor-binding protein	[77]
Anti-cancer antibiotics					
Daunorubicin	2-D-MALDI/TOF-MS	EPP85-181P		T-complex protein subunit α, transfer RNA-Trp-synthetase, T-complex protein subunit β, cytokeratin19, cytokeratin7, elongation factor 1χ, heterogeneous nuclear ribonucleoprotein, erythrocyte cytosolic protein of 51 kDa, differentiation-related gene product, heat shock protein 60, glucose regulated protein of 78 kDa Grp 78, heat shock 27 kDa protein	[78]
Doxorubicine	2-D-MALDI/TOF-MS	MCF-7	Macrophage migration inhibitory factor	heat shock 27 kDa protein	[79]
Antimetabolites					
5-Fluorouracil	2-D-MALDI/TOF-MS	HeLa	Glutathione-S-transferase, MAP kinase 13, amino-methyltransferase, eukaryotic translation initiation factor 3 subunit 3 and 4H, protein kinase C inhibitor protein-1, heterogeneous nuclear ribonucleoproteins C1/C2, homeobox protein BarH-like 2, ubiquitously expressed transcript protein, cell death activator CIDE-B, NADH-ubiquinone oxidoreductase, PDGF-associated protein, serine–threonine protein phosphatase 2B catalytic subunit, adenosylsuccinate synthase, acyl-CoA dehydrogenase, telomeric repeat-binding factor 2, histone H1.3, cation-dependent mannose-6-phosphate receptor, Apo- 1/CD 95 (Fas), caspase-3, caspase-8, and NADP-dependent leukotriene B4 12-hydroxy dehydrogenase	Mitotic checkpoint protein BUB3, 60S ribosomal protein L3, nuclear ubiquitous casein and cyclin-dependent kinase substrate, cytohesin 3, myc proto-oncogene protein, endoplasmic reticulum protein ERp29 precursor, translation initiation factor eIF-2B, D-β-hydroxybutyrate dehydrogenase, protocadherin α 5 precursor, snRNA-activating protein complex 43-kDa subunit, Src substrate cortactin, transforming protein P21A	[128]
5-Fluorouracil + Cisplatin	2-D-MALDI/TOF-MS	HeLa	Cullin homolog 4B, pre-mRNA-splicing factor PRP17 (cell division cycle 40 homolog), engulfment and cell motility protein 2, RAC-beta serine/threonine protein kinase, TRAF family-associated NF-jB I-activator, G-substrate, peptidyl-prolyl cis–trans isomerase E, synaptosomalassociated protein 29, glutathione-s-transferase, mitogen-activated protein kinase 13, amino-methyltransferase, eukaryotic translation initiation factor 3 subunit 3, eukaryotic translation initiation factor 4H, protein kinase C inhibitor protein-1, heterogeneous nuclear ribonucleoproteins C1/C2, homeobox protein BarH-like 2, UXT protein, cell death activator CIDE-B, NADH–ubiquinone oxidoreductase 42-kDa subunit, NADP-dependent leukotriene B4 12-hydroxy dehydrogenase, PDGF-associated protein, serine–threonine protein phosphatase 2B catalytic subunit, adenylosuccinate synthetase, acyl-coA dehydrogenase and telomeric repeat-binding factor 2	Regulator of G-protein signaling 13, ubiquitin carboxyl-terminal hydrolase 14, mitotic spindle assembly checkpoint protein MAD2B, 60S ribosomal protein L3, mitotic checkpoint protein BUB3, nuclear ubiquitous casein and cyclin-dependent kinase substrate, cytohesin 3 (ARF nucleotide-binding site opener 3), translation initiation factor eIF-2B b subunit, myc proto-oncogene protein, protocadherin a-5 precursor, snRNA-activating protein complex 43-kDa subunit, D-b-hydroxybutyrate dehydrogenase, endoplasmic reticulum protein ERp29 precursor, squamous cell carcinoma antigen 2 (leupin), src substrate cortactin (oncogene EMS1), and transforming protein P21A (c-K-ras).	[129]
Others:					
Cisplatin	2-D-MALDI/TOF-MS	HeLa	G-substrate, NADPH: nicotinamide adenine dinucleotide phosphate-dependent carbonyl reductase 3, serine/threonine/tyrosine interacting protein, cytochrome P450 4A11 precursor, regulator of G-protein signaling 13, TRAF-interacting protein, zinc finger protein 90, peptidyl-prolyl cis–trans isomerase E, heterogeneous nuclear ribonucleoprotein A1, partitioning defective-6 homolog a, prothrombin precursor, and cyclin-dependent kinase inhibitor p27 (p27Kip1)	Tumor necrosis factor α-induced protein 3, myc proto-oncogene, ubiquitin carboxyl-terminal hydrolase 14, potassium voltage-gated channel subfamily G member 1 aldose reductase, mitotic spindle assembly checkpoint protein MAD2B, Rho guanine nucleotide exchange factor 6, aldose reductase, proliferating cell nuclear antigen, and mitochondrial processing peptidase α subunit	[130]

(*continued on next page*)

Table 2 (*continued*)

Anti-cancer agents	Methods	Cell lines	Up-regulated proteins	Down-regulated proteins	Ref.
Cisplatin	SILAC-SDS/PAGE-MALDI/TOF-MS	Jurkat T cells	Actin, adenine phosphoribosyltransferase, elongation factor 2, eukaryotic initiation factor 4A-I, microsomal signal peptidase 25 kDa, p21-Rac2, peroxiredoxin 1, phosphomevalonate kinase, pre mRNA splicing factor SRp30C, RNA-binding protein 4, tetratricopeptide repeat domain 9C, transmembrane protein 33, tubulin α-ubiquitous chain, tubulin β	Core-binding factor beta subunit, heterogeneous nuclear ribonucleoprotein G, heterogeneous nuclear ribonucleoprotein K, heterogeneous nuclear ribonucleoprotein I, heterogeneous nuclear ribonucleoprotein H, heterogeneous nuclear ribonucleoproteins A2/B1, interleukin enhancer binding factor 2, lamina-associated polypeptide 2 isoforms β/χ, 54 kDa nuclear RNA- and DNA-binding protein, probable rRNA processing protein EBP2, Rho GDP-dissociation inhibitor 2, spectrin α chain	[83]
Cisplatin	2-D-MALDI/TOF-MS	A431	14-3-3 ξ/δ and ε, heat shock cognate 71 kDa protein, tropomyosin α 3 and 4 chain, F-actin capping protein, actin, microtubule associated protein RP/EB1, annexin V, proteasome activator complex, 60 kDa heat shock protein	Calmodulin, heat shock cognate 71 kDa protein, protein disulfide isomerase A3, stathmin, peroxiredoxin, peptidyl-prolyl cis–trans isomerase	[131]
Bohemine	2-D-MALDI/TOF-MS-ESI-MS/MS	CEM		α Enolase, triosephosphate isomerase, eucaryotic initiation factor 5A, α and β subunits of Rho GDP-dissociation inhibitor 1	[85]
Bohemine	2-D-MALDI/TOF-MS	A549	α-enolase, phosphoglycerate mutase, triosephosphate isomerase, annexin I, annexin IV, actin, cytoplasmic 1, PDZ and LIM domain protein I, endoplasmic reticulum protein disulfide isomerase A3 (ER60), 60 kDa heat shock protein, mitochondrial	Glucose-6-phosphate 1-dehydrogenase G6PD, isomerase A3	[84]
Bohemine	LC-PF2D-MALDI/TOF-MS	CEM	60 kDa heat shock protein	crk-like protein, Histone H3.3, nuclease sensitive element binding protein 1, Histone H2B.a,	[51]
Trichostatin A	2-D-MALDI/TOF-MS	PaCa44	Programmed cell death protein (TFAR19), stathmin, chromatin assembly factor 1 subunit C, growth factor receptor bound protein 2, cytochrome c oxidase polypeptide Vb, ARP2/3 complex 16 kDa subunit, programmed cell death protein 5, peroxiredoxin 1, UEV protein (ubiquitin conjugating E2 enzyme variant), thioredoxin, hint protein, deduced protein product shows significant homology to coactosin	Nucleophosmin, translationally-controlled tumor protein, 60 kDa heat shock protein, Tropomyosin α 4 chain, calreticulin precursor, tropomyosin α 3 chain, heterogeneous nuclear ribonucleoproteins A2/B1, glyceraldehyde 3-phosphate dehydrogenase, ATP synthase beta-chain, 60S acidic ribosomal protein PO	[132]
Gemcitabine + Trichostatin A	2-D-PAGE-MALDI/TOF and Q/TOF-MS	T3M4			[133]
Trichostatin A			S100A11 protein, peroxiredoxin I, guanine nucleotide-binding protein β subunit-like protein	Stress 70 protein mitochondrial	
Gemcitabine			S100A11 protein, peroxiredoxin I	Stress 70 protein mitochondrial, tubulin β chain	
Trichostatin A + Gemcitabine			Peroxiredoxin I, superoxide dismutase, thioredoxin, glutathione-S-transferase, 14-3-3 protein α, stathmin	heat shock protein HSP 90-alpha, stress 70 protein mitochondrial, cytokeratin type II CK8	

associated with serum protein profiling or quantification and improve knowledge about cancer tissue and its secretome.

The identification of new diagnostic or prognostic biomarkers characterising risk of cancer development and/or tumor progression or drug targets is mostly based on direct analysis of cancer cells and tissues and often utilises some of the above described proteomic methods. Large-scale proteomic analysis of 64 human ovarian cancer tissue samples presenting benign, borderline and malignant stages based on histological evaluation was performed using DIGE with each sample run in duplicate. Evaluation of protein quantitative data using non-supervised clustering distinguished between normal and malignant tissue samples, however, application of supervised methods allowed objective classification into four groups – normal ovaries, benign, borderline and malignant. Additionally, protein spot changes significantly contributing to this classification were reported to represent potential diagnostic tumor markers. Among those candidates, cytokeratin 19-type1, ErbB3, prohibitin, adenylosuccinate lyase and integrin alpha-5 were verified via tissue microarrays and others are currently under development to be tested for diagnostic purposes in tissue as well as blood samples [69]. Application of DIGE with large format of 2-D electrophoretic separation for laser microdissected tissue was utilised by group of Kondo [70] to investigate proteomic signatures of several types of malignancies including lung, pancreatic, colorectal, hepatocellular and cholangiocellular carcinomas, malignant mesotheliomas and soft-tissue sarcomas. Interestingly, they identified 43 protein spots whose intensity was statistically different between gastrointestinal stromal tumor (GIST) with good and poor prognosis. Mass spectrometric protein identification showed eight of the 43 spots derived from pfetin, a potassium channel protein, and four of the eight pfetin spots had a high discriminative power between the two groups. Western blotting and real-time PCR showed that pfetin expression and tumor metastasis were inversely related. The prognostic performance of pfetin was also examined by immunohistochemistry on 210 GIST cases. The 5-year metastasis-free survival rate was 93.9% versus 36.2% for patients with pfetin-positive versus pfetin-negative tumors, respectively ($P < 0.0001$). Univariate and multivariate analyses revealed that pfetin expression was a powerful prognostic factor among the clinicopathologic variables examined, including risk classification and c-kit- or platelet-derived growth factor receptor A mutation status. These results may also provide novel therapeutic strategies to prevent metastasis of GIST [71].

To obtain new insight into cancer cell invasion and migration, multidimensional protein identification technology (MudPIT) has been used in six established ovarian cancer cell lines. Within whole cell extracts MudPIT identified proteins that mapped to 2245 unique genes. Unsupervised cluster analysis partitioned the cell lines in a manner that reflected their motile/invasive capacity. A compar-

ison of protein expression profiles between cell lines of high versus low motile/invasive capacity revealed 300 proteins that were differentially expressed, of which 196 proteins were significantly up-regulated in group with high invasive capacity. Western blot analysis for selected proteins confirmed the expression profiles revealed by MudPIT, demonstrating the fidelity of this high-throughput analysis. Protein network modeling indicated a functional interplay between proteins up-regulated in group of high motile/invasive capacity cell lines characterised and increased expression of several key members of the actin cytoskeleton, extracellular matrix and focal adhesion pathways. These proteomic expression profiles could prove to be essential in the development of more effective strategies that target pivotal cell signalling pathways used by cancer cells during local invasion and distant metastasis [72].

Many cancer patients produce antibodies against tumor-associated antigens. The humoral immune response represents a form of biological amplification of signals that are otherwise weak because of very low concentrations of antigen, especially in the early stages. Hence, it is interesting to employ protein microarrays to identify these antigens. In one typical study, protein microarrays containing 5005 human proteins and auto-antibodies from 30 cancer patients were used to identify proteins that are aberrantly expressed in ovarian tissue. The differential reactivity of four antigens was tested by using immunoblot analysis and tissue microarrays. Lamin A/C, structure specific recognition protein 1 (SSRP1), and Ral binding protein 1 (RALBP1) were found to exhibit increased expression in the cancer tissue relative to controls. The combined signals from multiple antigens proved to be a robust test to identify malignant ovarian tissue and proteins aberrantly expressed in different disease states [73].

6. Focus on prediction of efficacy and monitoring of anti-cancer therapies

Clinical importance of biomarkers suitable for monitoring therapeutic response is of growing importance. Assessment of an individual patient and tumor predisposition to drugs is prerequisite to successful application of targeted anti-cancer therapy. This approach is needed namely for clinicians to stratify the patients and make proper decision about chemotherapeutic drugs that would be most effective and with minimal side effects. It is expected that proteomic profiling will help to answer the questions of tumor drug sensitivity/resistance and knowledge of molecular mechanisms that underly cancer drug response would significantly contribute to achieving this aim. In order to predict cancer drug response by proteomic profiling, Ma et al. [74] used reverse-phase protein microarrays covering 60 human cancer cell lines (NCI-60 panel) to measure protein expression levels by 52 antibodies. The specificity of each of the antibodies was verified by Western blotting. The NCI-60 panel contains 2–9 cell lines per each histological tumor type including leukemias, melanomas, and carcinomas of ovaries, renal, breast, prostate, colon, lung and central nervous system [75]. The drug activities of 118 agents were measured by growth inhibition within 48 h. In order to establish the classifiers that would be independent of tissue of origin, a combination of several algorithms were used. The results showed that it was feasible to used this data set (60 cell lines $\times$ 52 targets $\times$ 118 drugs) to generate significant chemosensitivity classifiers for 118 evaluated agents with statistical significance level $P < 0.02$. The range of drug responses was classified into three categories: sensitive, resistant and intermediate. It is important to stress, that this type of proteomic studies are focused on prediction of response without any analyses of molecular mechanisms underlying response to the drug.

The era of chemotherapy began in 1940s with the first uses of nitrogen mustards, which are nonspecific DNA alkylating agents, and later antifolates. In the early 1950s other anti-tumor drugs like anti-metabolites and vinca alkaloids came into clinical trials and use. In 1955 began systematic drug screening at the National Cancer Institute and new screening approach based on use of 60 cell lines derived from different human cancer types was introduced in 1989 [76]. The following are examples of several recent studies focused on more detail proteomic analyses of response to selected conventional chemotherapeutic drugs. The effect of alkylating agent temozolomide alone or in combination with radiotherapy on the protein expression profile of human U87-MG glioma cells in vitro was investigated by Trog et al. [77]. Using comparative analysis of protein expression pattern they found upregulation of vimentin, an extracellular matrix component, induced by temozolomide treatment. Interestingly, vimentin contributes to the mechanism of tumor progression supporting its pro-invasive and pro-angiogenic activity. Hence, potential molecular target, which in fact contributes to expansion of the cancer is promoted by treated glioma cells. This finding demonstrates usefulness of proteomic studies for uncovering possible mechanisms that interfere with successful therapy. Anti-cancer antibiotics are known to cause DNA damage (intercalation into DNA bases, interference with DNA unwinding via inhibition of topoisomerases I and/or II) and to affect tumor cell growth. 2-D gel electrophoresis was used to investigate the response of pancreatic tumor cells after exposure to daunorubicin. Identified up-regulated proteins after drug exposure participate in a variety of cellular processes probably due to activation of various signalling pathways in response to daunorubicin [78]. Human breast tumor cell line MCF-7 was used to investigate protein profile changes after doxorubicin treatment. Three isoforms of heat shock 27 kDa protein (HSP27) were found to be significantly decreased [79]. Interestingly, in investigation performed by Casado et al. [80] cellular response to plant vinca alkaloid vincristine on MCF7 cells also revealed vincristine dependent regulation of specific isoforms of anti-apoptotic protein HSP27. Since there was no effect on HSP27 mRNA they suggested that vincristine was implicated in the protein post-translational modification with up-regulation of Ser 82 phosphorylation. However, such data should be interpreted carefully, as HSP27 is highly abundant protein and it is one of the most frequently responsive protein molecules in human samples [81].

The use of combination of conventional chemotherapeutic agent vinblastine with targeted agent rapamycin (mTOR inhibitor) was studied at the protein level on the EA.hy926 endothelial cell line using 2-D gel electrophoresis coupled to MALDI mass spectrometry protein identification. Some of the regulated proteins were involved in the processes of angiogenesis, proliferation, migration and apoptosis. The authors verified proteomic data for several proteins using Western blots and also applied the computer modelling for generating a network of molecules that have common function and share similar target pathways to show that observed protein changes merged into synergistic antiangiogenic activity of vinblastine and rapamycin [82]. Platinum cytostatics belong to the group of potent and clinically important metal-based anti-cancer drugs. One of the most recent studies concerning molecular effects of cisplatin on cancer cells was published by Schmidt et al. [83]. The authors performed quantitative analysis of protein changes after cisplatin-induced apoptosis in Jurkat T cells to identify proteins related to the mechanism of cisplatin function. SILAC was used for quantitative proteome analysis of control versus apoptotic cells after cisplatin treatment. The time point for full activation of apoptosis was chosen for experiments. It was interesting that 8 of 26 identified apoptosis-related proteins contained at least one RNA-binding motif.

In our studies we considered selective cyclin-dependent kinase inhibitor (CDKI) as a promising anti-cancer drug. We applied complementary proteomic approaches to analyse responses of CEM T-lymphoblastic leukemia and A549 lung adenocarcinoma lines as representatives of haematological malignancy and solid tumor, respectively. Based on the evaluation of the protein maps and possible pathways relevant to responses to CDKI, the glycolytic enzymes, annexin IV and the crkl adaptor protein appear to be important targets. Collectively, our proteomic findings underline the importance of cell cycle control in both the cellular signaling and metabolic pathways. Initial confirmation studies focused directly on the crkl protein have proved the validity of the proteomic results, but the potential utility of CDKI warrants further study and validation using different types of tumor models [51,84,85].

The analysis of known cell signalling networks or pathways for a patient can be currently obtained from a biopsy specimen of given individual using reverse-phase protein microarrays. In this approach, the entire cellular proteome is immobilised and subsequent immunodetection reveals phosphorylation status of signalling proteins. Therefore it is suitable for monitoring of multiple pharmacodynamic biomarkers of response to molecular targeted agents. Boyd et al. [86] applied this protocol to examine phosphorylation status of 100 proteins in a panel of 30 breast cancer cell lines and showed distinct pathways activation in different subtypes that were not obvious from previous gene expression studies. It was shown that inhibitors of epidermal growth factor receptor and mitogen-activated protein kinase/extracellular signal-regulated kinase result in compensatory up-regulation of the phosphatidylinositol-3-kinase/Akt signalling pathway indicating that combined inhibition of both pathways should translate into synergistic tumor killing.

Many of the above mentioned studies were followed up at various time interval when apoptotic process occur. However, more interesting and specific would be the characterisation of earlier protein changes that precede the on-set of apoptosis. More studies on the molecular mechanisms underlying the effect of individual conventional, targeted and developmental chemotherapeutics, or their combinations are urgently needed. In fact, the number of publications covering this issue is low (Table 2). Furthermore, monitoring molecular mechanisms in earlier time intervals after drug exposure may be beneficial for detecting relevant proteins that are responsible for primary changes in signalling networks and subsequently lead later to irreversible anti-cancer processes including cell division cycle block, induction of apoptosis, and interference with cancer-related angiogenesis, invasion and metastasis.

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