Proteomics and its application to discover chemoresistant biomarkers in human cancer

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Abstract

Primary or acquired resistance to traditional cytotoxic chemotherapy remains a major obstacle to the successful management of patients in clinical oncology. The discovery of reliable predictive biomarkers of response to chemotherapy-based treatments, which would lead to individualized therapeutic strategies, is a major challenge in cancer biomarker research. Recent innovative high-throughput proteomic technologies appear as powerful tools for the discovery of novel biomarkers associated with chemotherapy resistance because they facilitate the simultaneous analysis of whole proteomes. The current review describes the plethora of existing mass spectrometry (MS)-based proteomic studies that have been conducted to investigate chemoresistance in human cancer, and emphasize putative biomarkers and protein expression profiles that may be useful in the prediction of clinical response to anticancer therapy. We focus on five fatal cancers (breast, prostate, ovarian, lung and esophageal) and for each cancer type the proteomic approaches utilized for the identification of the novel biomarkers are described. A short overview of both gel-based and gel-free mass spectrometry methods is presented along with a brief summary of the most current methods utilized for validation of putative biomarkers. Although the majority of the putative biomarkers presented in this review are very promising, they have been limited to the discovery stage and they still have to be further clinically validated. Follow-up research should focus on the design of careful prospective multicentre screening studies and large clinical trials in order to identify the biomarkers that work and bring them into clinical practice as soon as possible.

Keywords:

• proteomics • mass spectrometry • predictive biomarkers • chemoresistance

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Abbreviations: 2D-DIGE, two-dimensional difference gel electrophoresis; 2DE, two-dimensional polyacrylamide gel electrophoresis; DEP, differentially expressed protein; ELISA, enzyme-linked immunosorbent assay; ICAT, isotope-coded affinity tagging; IHC, immunohistochemistry; iTRAQ, isobaric tags for relative and absolute quantification; LC, liquid chromatography; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MDR, multidrug resistance; MS, mass spectrometry; nanoLC-MS/MS, nanoflow liquid chromatography coupled to tandem mass spectrometry; NSCLC, non-small cell lung cancer; qRT-PCR, quantitative real time polymerase chain reaction; siRNA, small interfering RNA; SELDI-TOF MS, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; WB, Western blot.

1. Introduction

Cancer is one of the leading causes of death among men and women. In 2008, about 12.7 million new cancer cases and 7.6 million cancer deaths (around 13% of all deaths) are estimated to have occurred across the world [1,2]. Despite tremendous progress in early detection, diagnosis and therapy, the burden of cancer is increasing with an estimated number of 13.1 million deaths expected in 2030 [2].

A major obstacle to successful treatment and better outcome in cancer patients is the occurrence of chemotherapy resistance [3]. Cancer cells can be already primary resistant to drug treatment from the beginning (intrinsic resistance), or they can acquire resistance during chemotherapy [4]. Another hallmark of cancer therapy besides unpredictable drug response is the severe systemic toxicity of the chemotherapeutic agents, which in some patients can cause serious side effects, even death [5]. Thus, the ability to determine, at an early stage, the clinical response of a cancer to a chemotherapeutic regimen could prevent exposure of patients to agents with serious or life-threatening side effects for no therapeutic gain. In addition, overcoming resistance to chemotherapy would result in a reduction of health care costs and patient and families costs [6]. The major challenge in this regard, is to elucidate the mechanisms of chemoresistance and to identify novel predictive cancer biomarkers that may help design personalized therapeutic strategies.

Resistance to chemotherapy is a multifactorial phenomenon and although many mechanisms of drug resistance are known such as drug efflux, DNA repair and altered survival and apoptotic signalling pathways, others have yet to be discovered [7,8]. Since the discovery of P-glycoprotein as a biomarker of multidrug resistance in 1970s by Juliano et al. [9] many studies have been conducted to elucidate the mechanisms underlying drug resistance and to identify predictive markers of therapy response. In most of these studies, techniques such as immunohistochemistry, Western blot and RT-PCR have been applied to associate protein and/or gene expression with resistance to anticancer treatments [10-14]. However, such studies require prior biological knowledge of the selected protein targets that are to be tested and hence, are unable to discover novel therapeutic targets or establish the relations between targets [15]. Nowadays, the advances in global analytical techniques, which enable the simultaneous analysis of the whole genome or proteome of chemoresistant cancer cells, overcome such limitations.

A large number of published studies have utilised large-scale genomic and transcriptomic approaches including comparative genomic hybridization (CGH), array-based CGH but also cDNA microarrays and differential display RT-PCR to study drug resistance [16-22]. Given that information based on the genome and the transcriptome does not best reflect the function of the proteins, which are the key functional entities in a cell, proteomics has been an important area of cancer research [23]. Many regulatory processes occur post-transcriptionally, such as post-translational modifications (PTMs) of proteins and alternative RNA splicing events among others, which cannot be inferred from the nucleic acid level [24]. Therefore, proteomics may reveal mechanisms involved in drug resistance by directly addressing the functional effectors of cellular, disease and treatment processes [25]. Current progress in proteomics has been largely due to recent advancements in mass spectrometry (MS) technologies, which have enabled high sensitivity and automation in protein identification. The numerous MS-based quantitative proteomic approaches, have enhanced proteomic profiling analysis not only in established cell lines, but also in complex tissues and biological fluids to identify protein expression changes associated with chemoresistance [26]. High-throughput proteomics have the potential to reveal

novel protein biomarkers that may predict the differential efficacy (benefit) of a particular therapy and allow clinicians to select the most appropriate treatment for individual patients [27].

In the next sections, the most current advances on the application of mass spectrometry (MS)-based proteomic technologies to discover putative biomarkers associated with chemotherapy resistance in human cancer are highlighted. In addition, both gel-based and gel-free mass spectrometry technologies are briefly discussed along with the methods for technical verification and clinical validation of the identified biomarkers.

2. Proteomic approaches in the discovery of predictive cancer biomarkers

2.2 Short overview of mass spectrometry (MS)-based proteomic technologies

During the past decade, a growing number of studies in cancer biomarker research have been published utilizing different mass spectrometry (MS)-based methods. The complete panel of proteomic technologies used in cancer proteomic research can also be applied to study therapy response in human cancers and to identify putative biomarkers associated with resistance to anticancer therapy. These proteomic techniques include gel-based MS methods, (usually 2DE coupled to MALDI-TOF-MS) and gel-free MS methods (one- or two- dimensional liquid chromatography coupled to tandem MS, LC-MS/MS). Recently, protein profiling using surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS technology has been successfully employed in biomarker discovery. Here we provide a short overview of the most widely used proteomic techniques however, there are a handful of excellent descriptive review articles discussing them in detail [27-30].

Gel-based MS proteomic methods are based on protein separation from a complex mixture using gel electrophoresis prior to protein identification by mass spectrometry (MS). Usually, complex protein samples are separated by two-dimensional gel electrophoresis (2DE); firstly, horizontally, by isoelectric focusing, which separates the proteins according to their isoelectric point, *p*I and then, vertically, according to their molecular weight using SDS-PAGE [31,32]. Proteins can be stained before 2DE separation (pre-electrophoretic stain), or after 2DE separation (post-electrophoretic stain) using several sensitive staining methods or fluorescent techniques [33]. Pre-electrophoretic protein stains are used in the two-dimensional fluorescence difference gel electrophoresis or difference gel electrophoresis (2D-DIGE) [34]. 2D-DIGE is nowadays an interesting alternative for the traditional 2DE, since it allows co-electrophoresis of two or three differentially labelled samples with fluorescent molecules (Cy2, Cy3 and Cy5) on a single 2DE gel and therefore, comparison of the samples is improved since gel-to-gel variations are minimized [35]. The differentially expressed protein spots identified by 2DE analysis are excised from the gel, in-gel digested into peptides by sequence-specific proteases (e.g. trypsin) and subsequently subjected to MS analysis for protein identification. In the recent years, two-dimensional gel electrophoresis (2DE) coupled to MALDI-TOF MS is commonly used for comparative proteome analysis in biomarker discovery [36]. Despite limitations such as poor resolving power for low abundant, acidic, basic, hydrophobic, very large or small proteins and difficulties in quantifying co-migrating proteins, 2DE-MS has generated many candidate biomarkers with a great clinical potential in various cancers [37].

The gel-free MS-based methods use liquid chromatography (LC)-based separation techniques directly coupled to automated MS/MS (LC-MS/MS). In shotgun proteomics (bottom up strategy) proteins of complex samples are specifically digested by proteases into peptides, and the complex peptide fractions are resolved in one or more dimensions of LC prior to identification by MS. The most commonly used two-dimensional LC separation, first described as multidimensional protein identification technology (MudPit), combines strong cation-exchange chromatography (SCX) for the first dimension and reverse phase chromatography (RP) for the second dimension coupled to tandem MS/MS [38]. LC-MS/MS strategies offer high-throughput analyses resulting in the acquisition of hundreds to thousands of MS/MS fragmentation spectra in a single experiment [39]. Unlike proteomic analysis by 2DE-MS, LC-MS/MS analysis is not a comparative, quantitative proteomic technique. However, recent advances in mass spectrometry methodologies have led to the development of label-based and label-free strategies for protein quantitation [40].

The labelling methods for quantitative proteomics use differential stable isotopes to label samples prior to the MS analysis. In vivo metabolic labelling by stable isotope labelling of amino acids in cell culture (SILAC), is used to label proteins in the cell culture prior to MS analysis by the addition of stable isotope containing amino acids (e.g. ^{2}H, ^{13}C, ^{15}N) in the cell culture medium [40]. Isotope-coded affinity tagging (ICAT) technology uses chemical reagents which consist of a biotin tag, a light or heavy deuterated linker and a thiol-specific reactive group that binds to the cysteine residues of the protein and can be used to label paired protein extracts [41]. Quantification using isobaric tags for relative and absolute quantification of peptides (iTRAQ) is achieved at the MS/MS level and allows the multiplex analysis of up to eight samples in the same experiment [42]. Finally, a straightforward, fast and cost-effective method for triplex quantification at the MS level is stable isotope dimethyl labelling [43]. It is based on the incorporation of isotopomeric dimethyl labels at the peptide level and can be applied in any sample type from primary cells to tissues and biological fluids [44].

In label-free LC-MS methods, protein quantification is achieved by comparing MS measurements of different samples. Label-free quantification through spectral counting is based on the principle that highly abundant peptides will generate a higher number of MS/MS spectra [45]. In other label-free methods the peak intensities of the detected peptides are used rather than the spectral counts to obtain quantitative information [46].

Protein profiling using surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) MS technology has gained over the past few years a considerable attention in the field of biomarker discovery [47] and has been applied in several studies for the discovery of predictive candidate biomarkers. SELDI-TOF MS couples array-based technology with MALDI-TOF MS. In SELDI, proteins and peptides present in complex biological samples are resolved by binding to biochemically distinct ProteinChip arrays [48]. These ProteinChip arrays, consist of a chemically (hydrophobic, cationic, anionic, hydrophilic or immobilized metal affinity) or biochemically (high affinity protein-protein interaction; antibody, antigen binding fragments such as scFv, DNA, enzyme or receptor) modified active surface [48]. After application of a matrix a ProteinChip SELDI Reader generates a mass profile by laser desorption/ionization time-of-flight mass spectrometry analysis. This technique usually generates a distinctive proteomic pattern based on differential mass spectra to distinguish between two different samples. Therefore, patterns of masses rather than actual identifications of protein biomarkers were deemed as the important factors in SELDI-TOF analysis [15]. However, with the advance of technology SELDI-TOF can now be combined with tandem MS to obtain sequence information and hence, identification of the protein of interest [15].

2.3 Validation of identified putative biomarkers

In cancer biomarker discovery, the success of the proteomic studies largely depends on the verification and clinical validation of the identified putative biomarkers. Global approaches such as high-throughput proteomics, which enable the simultaneous analysis of a large number of expressed proteins, are prone to false discovery and overinterpretation [27]. Therefore, technical validation of the proteomic data is necessary to confirm the accuracy, the precision and reproducibility of the analytical assay [49]. In most proteomic studies, a second independent technique such as Western blotting, ELISA and/or immunohistochemistry, which may be combined with a complementary RNA-based transcriptomic screening approach, is used to confirm the identified biomarker and the detected differential protein expression not only in the analysed sample set, but also in independent sample sets [24,50]. Once a candidate protein biomarker has successfully passed through validation by the aforementioned experiments then, further detailed studies including a functional study and clinical validation in a sufficient number of suitable clinical samples from cancer patients can ensue. Functional studies usually make use of pharmacological inhibitors, antisense RNA or RNA interference (RNAi) methods to silence gene expression [50].

Recently, selected reaction monitoring (SRM), also called multiple reaction monitoring (MRM), has emerged as a promising mass spectrometry technique for validation of protein expression changes [51]. SRM assays allow reliable, fast, reproducible and cost-effective identification and quantification of dozens of selected promising protein biomarkers in a wide variety of clinical samples [52]. In cases in which a large number of potential protein biomarkers are to be tested, SRM is preferred rather than antibody-based approaches, which are very expensive. In addition, SRM is an excellent alternative method when suitable antibodies for Western blot, ELISA and IHC analyses are not available [24].

3. Predictive biomarkers of response to chemotherapy identified by comparative proteomics in cancers

The ability to predict sensitivity of a tumor to chemotherapeutic drugs would allow chemotherapy administration to be directed towards cancer patients who would most likely benefit and thus, prevent non-responder patients to experience the burden of ineffective and potentially toxic treatments [15]. Many comparative proteomic studies have been conducted to elucidate mechanisms by which cancer cells acquire drug resistance and to identify potential predictive biomarkers using various drug selected human cancer cell lines [53-60]. By these studies, long lists of differentially expressed proteins that might play a role in chemoresistance were published [8]. The identified proteins belong to a wide range of different classes of proteins including calcium-binding proteins, cell cycle and checkpoint proteins, chaperones and DNA repair proteins, cytoskeletal proteins, metabolic enzymes, several ribosomal proteins, transmembrane proteins as well as proteins involved in the cellular redox system and in signal transduction (detailed overview of these studies in ref [8]).

The following text describes representative results of the most recent MS-based proteomic studies focused on identifying novel predictive biomarkers of chemotherapy response in several human cancers. In these studies both cultured cell lines and clinical samples (tumor tissue, biological fluids) were used, while a limited number of published studies investigated the protein expression pattern in tumor xenografts derived from human cancer cell lines growing in

mice. Identified putative predictive biomarkers and the proteomic approaches employed for the discovery are summarized in Table 1. The list has been limited mainly to biomarkers that have been validated by one or more independent methods, ranging from Western blot to immunohistochemistry or functional analyses.

3.1. Breast cancer

In breast cancer, several proteomic studies have been performed to identify predictive biomarkers of tumor response to chemotherapy. Thus far, the majority of published studies have been mainly performed in cultured breast cancer cell lines [27]. These studies require the establishment of new drug-resistant cell sublines to identify differentially expressed proteins (DEPs) by comparing their proteome to that of the parental cell line [27]. To date, proteomic analysis of chemotherapy-resistant breast cancer cell sublines derived from luminal-type (ER-positive) MCF-7 parent cells [61], by 2DE couple to MS has led to the identification of large number of differentially expressed proteins [62-64].

In a comprehensive study, Liu and co-workers studied adriamycin resistance in breast cancer cells [65]. Adriamycin, also known as doxorubicin, is an anthracycline antibiotic, which works by intercalating DNA [66]. Adriamycin remains an important agent in various chemotherapy regimens and is commonly used in the treatment of breast cancer [67]. Liu et al. compared the global protein profiles between adriamycin-resistant MCF/AdVp3000 cells and the parental cell line MCF7, using 2DE and MALDI-TOF MS. In total, 17 proteins with differential expression levels were identified. Of these, 14-3-3 sigma (stratifin) protein, which was overexpressed in the drug-resistant cell subline, was selected for further validation by Western blot analysis and real-time quantitative RT-PCR. To validate the importance of 14-3-3 sigma as a biomarker of adriamycin resistance, they performed an experiment to knock down 14-3-3 sigma expression in MCF/AdVp3000 cells. Indeed, siRNA transfected cells showed a decrease in the drug resistance level [65]. Interestingly, 14-3-3 sigma has also been associated with multi-drug resistance (MDR) in MCF breast cancer cells exposed to 5-fluorouracil chemotherapy by Zheng et al. [68].

The taxanes (paclitaxel and docetaxel) are antimicrotubule anticancer agents, which are currently used as first-line chemotherapy for the treatment of breast cancer [69,70]. In many women with locally advanced breast cancer, taxane-based chemotherapy is widely used as an adjuvant to surgical removal. Bauer et al. [71] applied proteomics to search for predictive markers of response to neoadjuvant paclitaxel/radiation in breast cancer patients in tumor tissue obtained from pre-treatment biopsies. Patients were treated with three cycles of paclitaxel followed by concurrent paclitaxel/radiation. Next, proteomic analysis of tissues obtained from pre-treatment biopsies from patients that achieved a pathologic complete response (pCR) and from those with residual disease (non-pCR) followed. Results revealed that pre-treated breast tumors from patients with a pCR showed an overexpression of α-defensins (DEFA). Immunohistochemistry analysis was performed to validate the overexpression of DEFA as a potential marker of response to neoadjuvant paclitaxel/radiation in breast cancer [71].

In another study He et al., used surface-enhanced laser desorption ionization/time-of-flight (SELDI-TOF) MS technology, to explore whether tumor proteomic profiling prior to neoadjuvant chemotherapy could predict tumor response to therapy. The chemotherapy regimen was Taxotere/Carboplatin ± Herceptin. SELDI mass spectra were obtained from tumors with various sensitivities to treatment and results suggested that it might be feasible to pre-select patients for optimal chemotherapy since a difference of a single peak intensity at m/z 19906 correctly separated 88.9% of the tumors with pCR and 91.7% of the resistant tumors (non-pCR) [72].

Tamoxifen is an antiestrogenic agent that is widely used in the treatment of patients with breast cancer despite the high percentage of women who are (or become) resistant to this treatment [73]. Moreover, tamoxifen-resistance is considered the major cause of death in patients with recurrent breast cancer and thus, identification of biomarkers that could predict therapy response is of crucial importance. To this end, a comparative proteomics approach on tamoxifen therapy-sensitive and therapy-resistant breast tumor cells obtained through laser capture microdissection (LCM) was performed using a nano-LC-FTICR MS technology. A panel of putative protein biomarkers (extracellular matrix metalloproteinase inducer (EMMPRIN), ENPP1, EIF3E and GNB4) indicative of tamoxifen therapy resistance in breast cancer were identified. Validation of the top discriminating protein, EMMPRIN, in an independent patient cohort by immunohistochemistry using high density breast tissue microarrays confirmed its correlation with poor outcome on first line tamoxifen treatment in recurrent breast cancer [74]. Besada et al. used a proteomics approach to compare the proteomes of tamoxifen-resistant and tamoxifen-sensitive xenografted breast tumors [75]. Results of the proteomic analysis showed that twelve proteins were up-regulated in the tamoxifen-resistant line, whereas nine were down-regulated. Among the identified proteins, ALG-2 interacting protein and two GDP-dissociation inhibitors might serve as novel biomarkers for predicting tamoxifen response, however, their involvement in the drug-resistance phenomenon has yet to be determined as well as their validation in patient derived tumor samples. Interestingly, down-regulation of ALG-2 interacting protein has also been described by Arzu et al. [74]. However, the list of the other differentially expressed proteins identified in this study did not overlap with the list reported by Arzu and co-workers.

An interesting comprehensive quantitative proteomic study to identify predictive biomarkers of resistance to neoadjuvant chemotherapy with doxorubicin and docetaxel was performed on needle–biopsied tissues of patients with locally advanced breast cancer [76]. Breast cancer tissues were collected prior to chemotherapy and subsequently analysed by LC-MS/MS. Quantitation was based on spectral counting and the mass spectrometric data were confirmed by Western blot analysis. Clinical validation by immunohistochemistry analysis of the proteins FKBP4 and S100A9 demonstrated that based on their expression levels, it might be possible to classify breast cancer patients in drug-sensitive and drug-resistant groups prior to treatment with a combined chemotherapy using doxorubicin and docetaxel. In particular, FKBP4 was highly expressed in patients' samples with a poor response to chemotherapy (drug-resistant group). On the other hand, S100A9 was expressed in low levels in the drug-resistant tissue samples whereas a strong staining was observed in the drug-sensitive samples.

In summary, proteomic studies in breast cancer have revealed a number of promising proteins that might serve in the future as predictors of response to chemotherapy. Although for most of the candidate biomarkers retrospective validation is presented, these markers await further clinical validation in prospective multicentre studies.

3.2 Prostate Cancer

The majority of deaths from prostate cancer (PCa) are driven by the development of metastatic disease. Docetaxel, a taxane chemotherapeutic, remains the standard front-line treatment in patients with metastatic prostate cancer [77,78]. Even though docetaxel is currently the most effective drug used in the treatment of patients with advanced hormone-refractory prostate cancer (HRPC), approximately 50% of HRPC patients will not respond to docetaxel-based therapy and will develop resistance [77].

To identify potential plasma/serum biomarkers that might predict response to docetaxel in HRPC, Zhao et al. [79] used iTRAQ-mass spectrometry analysis to compare the proteomes of docetaxel-sensitive PC3 cells and docetaxel-resistant PC3-Rx prostate cancer cells. Since they were interested in identifying plasma and/or serum biomarkers they focused on differentially expressed (DEPs) secreted proteins. Amongst 85 DEPs found in PC3-Rx cells in comparison to PC3 cells, only seven were secreted proteins. Of these 7 proteins, the authors selected MCI-1 and AGR2 to further assess their role in docetaxel resistance. Western blot analysis confirmed the differential expression of MCI-1 and AGR2 proteins, which were up-regulated and down-regulated in the drug resistant cells, respectively. When PC3 cells were transfected with a recombinant human MIC-1, rhMIC-1, a significant increase of docetaxel resistance in these cells was observed. In addition, when the docetaxel-resistant PC3-Px cells were transfected with MIC-1 siRNA they showed an increased sensitivity compared to the PC3 cells transfected with control siRNA. Accordingly, knock down of AGR2 expression in PC3 cells with AGR2-targeted siRNA promoted docetaxel resistance. Furthermore, an increase of MIC-1 level in serum/plasma samples collected from patients with HRPC after cycle 1 of docetaxel was associated with poor survival [79]. Hence, MIC-1 may predict for docetaxel resistance in the clinical setting and provide indication to abandon further chemotherapy in men with HRPC [79].

In a recently published research, O'Connell et al. [80] studied the proteome of four docetaxel-sensitive prostate cancer cell sublines as well. LC-MS analysis revealed 4 proteins with concurrent changes in protein abundance in all resistant cell lines: the heat shock protein HSP70, which was up-regulated and the cytokeratin K2C8, D-3-phosphoglycerate dehydrogenase (SERA) and Valyl-tRNA synthetase (SYVC), which were down-regulated. Moreover, docetaxel resistance in prostate cancer was associated with changes in expression levels of a number of HSP and cytoskeletal proteins. Validation by Western blot of the identified proteins confirmed the proteomic data. The altered expression levels of K2C3 and SERA are also in agreement with the findings of Zhao et al. [79].

To explore the changes in protein expression associated with acquired in vivo resistance towards metronomic cyclophosphamide (CPA) treatment, Thoenes et al. compared CPA-resistant prostate cancer cell sublines obtained from in vivo resistant xenograft tumors to the non-resistant parental cell line. Out of a total of 25 differentially regulated proteins, the authors verified the association of annexin A3 with metronomic CPA resistance by Western blot analysis both in vitro and in xenografts. Moreover, an increase in the expression levels of upon metronomic CPA therapy of tumor-bearing mice was observed specifically in the resistant tumors and not in the chemosensitive control tumors [81].

So far, a limited number of proteomic studies have been conducted to study chemotherapy resistance in prostate cancer. However, promising biomarkers have been detected mainly from the analysis of cultured prostate cancer cell lines. In the future, further extensive studies are required to indicate the potential of these proteins as biomarkers of drug resistance in prostate cancer and/or drug targets for therapeutic intervention.

3.3 Ovarian Cancer

Due to the absence of specific symptoms in the early stage, the majority of women with ovarian cancer are diagnosed at an advanced disease stage. Combination chemotherapy is the standard treatment for nearly all women diagnosed with ovarian cancer however, despite high initial response rates, most patients will relapse after achieving complete clinical response [82].

To discover biomarkers indicating drug resistance to paclitaxel in ovarian cancer cells, Lee and co-workers established four different paclitaxel-resistant sublines, SKpacs, from the

sensitive parental human epithelial ovarian cancer cell line, SKOV3 [83]. Quantitative proteomic analysis using 2DE coupled to mass spectrometry (MALDI-TOF and LC-MS/MS) revealed ALDH1A1, annexin A1, hnRPN A2 and GDI 2 proteins to be differentially expressed in the resistant cell lines. Moreover, the expression of these candidate proteins was further validated by Western blotting not only in the chemoresistant cell lines but also in chemoresistant ovarian cancer tissues. The authors suggested that down-regulation of hnRNP A2 and GDI 2 proteins might predict paclitaxel resistance in ovarian cancer [83].

Cicchillitti et al. compared the proteome of the human epithelial ovarian cancer cell line A2780 to its paclitaxel-resistant subline A2780TC1 [84]. Analysis by differential in-gel electrophoresis (2D-DIGE) coupled to mass spectrometry (MALDI-TOF and LC-MS/MS), identified the disulphide isomerase ERp57 protein to be overexpressed in the resistant cells. The authors validated the differential expression by Western blot analysis and suggested ERp57 as a predictive biomarker of paclitaxel resistance in ovarian cancer. Moreover, their data suggest that ERp57 is involved in the paclitaxel resistance through its interaction to β-tubulin (TUBB3), which is a clinical marker of drug resistance in ovary [84].

A quantitative proteomics approach based on iTRAQ-LC-MS/MS to was employed to identify differentially expressed proteins between a cisplatin-resistant ovarian cancer cell line and its parental cisplatin-sensitive cell line that may influence resistance to chemotherapy. In total 28 DEPs were identified and a number of them were validated by Western blot analysis and/or qRT-PCR. Of those, the most promising were the PK-M2 and HSPD1 proteins, which were significantly down-regulated and up-regulated in the resistant cells, respectively. Their role to cisplatin-resistance was further confirmed by siRNA transfection [85].

From the results of the studies presented, proteins involved in important biological processes including chaperones (HSPD1, Erp57), metabolic enzymes (ALDH1A3, PK-M2), a member of the annexin superfamily of proteins, the heterogeneous nuclear ribonucleoprotein, hnRNP 2 and the Rho GPD dissociation inhibitor, GDI 2 have been associated with resistance to anticancer therapy in ovarian cancer cells. Unfortunately, no prospective clinical trials have been conducted to clinically validate the value of these biomarkers in ovarian cancer patients.

3.4 Lung Cancer

Non-small cell lung cancer (NSCLC) represents 85% of lung cancer cases and is frequently diagnosed at an advanced disease stage or when the disease has already spread with distant metastasis [86,87]. At these late stages, combination chemotherapy is the standard treatment modality for NSCLC patients. However, non-small cell lung cancer shows a strong primary resistance to anticancer drugs and only a subset of patients (30 to 35%) benefit from any particular chemotherapeutic regimen [52]. Therefore, to identify predictive lung cancer biomarkers which could guide therapeutic decisions and lead towards individualized therapies for NSCLC patients in the future is of crucial importance.

Although the antimetabolite gemcitabine is considered one of the most-effective drugs used in NSCLC chemotherapy, many patients experience treatment failure due to the occurrence of drug resistance [88,89]. To identify protein markers for gemcitabine-resistance Qu et al. [90] studied the differences in the proteomes of gemcitabine-sensitive and gemcitabine-resistant NSCLC cell lines by applying a quantitative proteomic approach based on isotope-coded affinity tag (ICAT) and LC-ESI-MS/MS. Amongst 14 identified DEPs in the gemcitabine-resistant sublines, the overexpressed calcium-binding protein sorcin, was selected and further validated by Western blot and IHC analysis on NSCLC tumor tissues. Thus, they suggested that sorcin may be a potential predictive biomarker of gemcitabine resistance in NSCLC [90].

Proteomic techniques have also been adopted to investigate biomarkers associated with cisplatin-resistance in NSCLC. Cisplatin is an antitumor agent that binds to DNA and forms intrastrand DNA crosslinks, which induce DNA damage, inhibition of DNA synthesis, RNA transcription suppression, effects on the cell cycle and apoptosis [91]. In the study of Zeng et al. [92], a proteomic analysis was performed using 2DE combined with MALDI-TOF mass spectrometry. Comparison of protein expression profiling between a cisplatin-sensitive cell line and its cisplatin-resistant subline identified found DJ-1 to be significantly up-regulated in the resistant cells. The differential expression of DJ-1 between the cisplatin-resistant lung cancer cells and the sensitive parental cells was confirmed by WB analysis demonstrating the validity of the proteomic data. To further validate the association of DJ-1 overexpression with the development of cisplatin resistance in NSCLC patients, immunohistochemical analysis was performed on 67 locally advanced NCSLC tumor tissues. Results showed that DJ-1 overexpression was indicative of cisplatin-resistance and that the group of patients with DJ-1-high expression levels had a significantly shorter survival time compared to the DJ-1-low group. In addition, silencing of DJ-1 by siRNA decreased cisplatin resistance in the resistant cells [92].

Recently, a similar proteomic study by Kuang et al. demonstrated the increased expression of dihydrodiol dehydrogenase 2 (DDH2) as a promising serum biomarker that might provide prediction of therapeutic response to cisplatin in advanced NSCLC patients [93]. A 2DE coupled with MALDI TOF-MS/MS comparative proteomic analysis between the cisplatin-resistant A549/DDP cell line and the parental A549 cell line, revealed eight significantly differentially expressed proteins. The secreted protein DDH2 was chosen by the authors as the protein of interest and its expression was validated by WB, real-time PCR and IHC analyses not only in cultured cell lines but in tumor xenografts as well. In addition, this potential serum biomarker protein has been further verified by ELISA analysis from 105 NSCLC patients treated with cisplatin-based chemotherapy. ELISA data showed an association of DDH2 expression with the clinical response to cisplatin based doublet chemotherapy.

Han et al. [94] employed SELDI-TOF MS technology, resulting in a specific proteomic profile that might be used to predict chemotherapy resistance amongst patients with advanced NSCLC. Using SELDI-TOF-MS they compared the protein spectra of serum samples obtained from chemotherapy sensitive and chemotherapy resistant patients. The chemotherapy regimen was a combination of cisplatin and docetaxel and the analysed serum samples were collected prior to and after treatment. Interestingly, they reported a five-peak pattern with m/z values at 3955 Da, 6207 Da, 7992 Da, 9214 Da and 15,086 Da, which could successfully distinguish the chemotherapy resistant group from the sensitive group. In addition, the predictive pattern showed 83.3% and 85.7% of sensitivity and specificity, respectively.

In NSCLC cancer, proteomic research on predictive biomarkers has led to the discovery of promising protein candidates. Amongst them, DJ-1 and DDH2, which have been extensively retrospectively validated. However, all putative biomarkers presented still lack a solid clinical validation to be further applied in clinical practice.

3.5 Esophageal cancer

In esophageal cancer (EC), which is histologically classified in two main types: esophageal squamous cell carcinoma (ESCC) and adenocarcinoma, several proteomic studies have been conducted to identify biomarkers that could predict tumor sensitivity to chemotherapeutic drugs.

To gain insights into the mechanisms of ESCC drug resistance, Wen et al. performed a comparative proteomic study between a cisplatin-resistant ESCC cell line and its counterpart

parental cell line [95]. The proteomic analysis was performed using 2DE coupled to MALDI-MS and in total 44 DEPs were identified. Among these, thioredoxin domain-containing protein 4 precursor and cystathionine gamma-lyase were selected as candidate biomarkers that might play a role in ESCC chemoresistance. In addition, further validation by Western blot and RT-PCR analyses of the two up-regulated proteins in the resistant cells confirmed the proteomic data.

In a recently published research, Langer et al. applied proteomics to identify biomarkers that could allow the prediction of chemotherapy response in patients with advanced esophageal adenocarcinoma. Pretherapeutic biopsies of patients treated by a neoadjuvant, 5-fluorouracil/cisplatin-based chemotherapy were analysed by an explorative proteomic approach. Radioactive labeling and high resolution two-dimensional electrophoresis was employed to detect and quantify the DEPs between the responders and nonresponders, while protein identification was achieved by MALDI-MS analysis. Comparison of the protein expression patterns identified cytoskeletal proteins and proteins belonging to the molecular chaperone family to be differentially expressed. The data of the proteomic analysis were confirmed by IHC and qRT-PCR. Interestingly, a significant correlation between therapy resistance and low expression levels of the heat-shock 27 protein (HSP27) was observed [96].

To discover novel serum predictive biomarkers in esophageal adenocarcinoma, proteomes of serum samples from human esophageal adenocarcinoma xenografts treated with epirubicin, cisplatin or 5-fluorouracil were analysed by SELDI-TOF MS. Proteomic comparison between treated and untreated xenograft mice, revealed a panel of peaks which reflected response to chemotherapy and subsequently fractionated and analysed by nanoLC-MS/MS for protein identification. Among those murine plasma proteins, serum amyloid A (SAA), transthyretin and apolipoprotein A-I were confirmed in clinical samples collected from patients with esophageal adenocarcinoma prior and after chemotherapy, demonstrating the validity of the proteomic approach employed. Particularly, serum amyloid A expression was decreased in the plasma samples after chemotherapy, whereas transthyretin and apolipoprotein A-I were increased [97].

In esophageal cancer, similarly to the aforementioned cancers, follow-up studies should be performed to assess the role and clinical value of the identified candidate biomarkers in chemotherapy resistance.

3.6 Other cancers

Cisplatin-based chemotherapy is the most widely used standard chemotherapeutic regimen for advanced and metastatic bladder cancer. It is estimated that patients show a clinical response rate of 50% however, the majority of these patients eventually experience disease recurrence [98]. In a recently published research, a comparative proteomic analysis of a cisplatin-resistant subline (HT1376-CisR) and its parental human bladder cell line HT1376 identified adseverin (SCIN) as a promising biomarker of chemoresistance in bladder cancer [99]. The overexpression of adseverin in the resistant cells was confirmed by WB analysis while SCIN silencing by siRNA transfection restored sensitivity to cisplatin [99].

Proteomics has also been applied to find predictive markers that might help in stratifying patients with acute myeloid leukemia (AML) to responders and non-responders to treatment. Albitar and co-workers analysed peripheral blood plasma from patients diagnosed with AML prior to induction chemotherapy using SELDI-TOF MS [100]. Distinctive peaks with a significant correlation with clinical response to therapy were identified. Decision tree algorithms were constructed by incorporating these peaks with various clinical and laboratory data (e.g. age, cytogenetic classification, blasts in peripheral blood etc.) to predict response. CART analysis showed that a peak at m/z 3223 when combined to age predicted responders of 83% accuracy,

while a peak at m/z 6611 when combined cytogenetic grouping along with the percentage of monocytes in peripheral blood, provided a prediction for responders with 95% accuracy [100].

A number of proteomic studies have been conducted by Yang and co-workers in order to elucidate mechanisms of chemoresistance in human gastric cancer [101,102]. According to the published data of these studies, proteomic comparison between a vincristine-resistant human gastric cancer cell line SGC7901/VCR and its parental cell line SGC7901 using 2DE coupled to mass spectrometry (MALDI-TOF MS and ESI-Q-TOF-MS), identified HSP27 and sorcin to be overexpressed in the vincristine-resistant cells. Functional experiments confirmed their contribution to the development of multidrug resistance (MDR) in SGC7901/VCR cells. In another comparative study, Yoo et al. [103] compared the protein profiles of human gastric cancer cell lines that were resistant to cisplatin or 5-fluorouracil (5-FU) and their parental cell lines, to identify proteins involved in drug resistance. The proteomic analysis was performed using 2DE and MALDI-MS and identified the pyruvate kinase isoenzyme M2 (PK-M2) to be expressed in low levels in the cisplatin-resistant cells compared to the parental cells. In addition, PK-M2 enzyme activity levels were significantly lower in most cisplatin-resistant cells and when both PK-M2 expression and activity were suppressed in the cisplatin-sensitive parental cells through antisense oligonucleotide transfection, these cells displayed increased drug resistance. Based on the presented data, the authors suggested that pyruvate kinase M2 is clearly related to cisplatin-resistance mechanisms. Interestingly, down-regulation of PK-M2 has also been reported as predictive biomarker of oxaliplatin resistance in patients with colorectal cancer [104]. A proteomic study by Shin and co-workers, conducted on human colon cancer cell lines with induced 5-fluorouracil resistance, identified the mitochondrial F(1)F(0)-ATP synthase to be down-regulated in the 5-FU-resistant cells providing clues for it as a promising predictive marker of 5-fluorouracil chemoresistance [105].

In pancreatic cancer, a 2DE analysis combined with LC-MS/MS was employed to investigate the differential expression of proteins between a gemcitabine-sensitive and a gemcitabine-resistant pancreatic cell line [106]. Results of the proteomic analysis revealed that the heat shock protein 27 (HSP27), was expressed in high levels in the gemcitabine-resistant cells. Moreover, HSP27 expression was verified by Western blot and by immunohistochemistry analysis in pancreatic cancer tissues. For support of these data, the resistant pancreatic cells were transfected by siRNA. Indeed, in the HSP27-silenced cells gemcitabine sensitivity was restored, suggesting that this protein contributes to gemcitabine resistance and might be a useful biomarker in predicting therapeutic response in pancreatic cancer patients.

Table 1. Putative biomarkers of therapy response identified by MS-based proteomics methods. The table includes the source used for biomarker discovery, the resistant drug and the techniques employed for the proteomic analyses and validation. Putative biomarkers demonstrated an increase (↑) or decrease (↓) in expression.

Putative biomarker	Source	Resistant drug	Proteomic Method	Validation	Ref.
Breast Cancer					
14-3-3 sigma (↑)	Cell lines	Adriamycin/ 5-Fluorouracil	2DE & MALDI-TOF-MS	Western blot qRT-PCR siRNA transfection	[65,68]
α-defensins (↑)	Tissue	Paclitaxel	Histology-directed MALDI- MS	IHC	[71]
Mass spectral profiles	Tissue	Taxotere/ Carboplatin ± Herceptin	SELDI-TOF MS	The predictors derived from the training set were used to predict chemo-responses in a validation set (n=17)	[72]
EMMPRIN (↑)	Tissue	Tamoxifen	Nano-LC-FTICR MS	IHC	[74]
ALG-2 interacting protein (↓) **GDP-dissociation inhibitor (↓)**	Tissue (xenograft)	Tamoxifen	2DE & ESI- Q-TOF-MS/MS	None	[75]
S100A9 (↑) **FKBP4 (↓)**	Tissue	Doxorubicin/ Docetaxel	LC-MS/MS	Western blot IHC	[76]
Prostate Cancer					
MIC-1 (↑) **AGR2 (↓)**	Cell lines	Docetaxel	iTRAQ, nanoLC-MS/MS	Western blot siRNA transfection	[79]
HSP70 (↑) **K2C8 (↓)** **SERA (↓)** **SYVC (↓)**	Cell lines	Docetaxel	nanoLC-MS/MS	Western blot	[80]
ANXA3 (↑)	Tissue (xenograft)	Cyclophospha-mide	2D-DIGE & MALDI-TOF-TOF	Western blot analysis in vitro and in xenograft-derived tumor tissues	[81]

Putative biomarker	Source	Resistant drug	Proteomic Method	Validation	Ref.
Ovarian Cancer					
hnRNP A2 (↓) **GDI 2 (↓)**	Cell lines	Paclitaxel	2DE & MS (MALDI-TOF & LC-MS/MS)	Western blot	[83]
ERp57 (↑)	Cell lines	Paclitaxel	2D-DIGE & MS (MALDI-TOF & LC-MS/MS)	Western blot	[84]
PK-M2 (↓) **HSPD1 (↑)**	Cell lines	Cisplatin	iTRAQ, LC-MS/MS	Western blot qRT-PCR siRNA transfection	[85]
NSCLC Cancer					
Sorcin (↑)	Cell lines	Gemcitabine	ICAT and LC-ESI-MS/MS	Western blot IHC	[90]
DJ-1 (↑)	Cell lines	Cisplatin	2DE & MALDI-TOF	Western blot IHC siRNA transfection	[92]
DDH2 (↑)	Cell lines	Cisplatin	2DE & MALDI TOF-MS/MS	Western blot qRT-PCR IHC ELISA	[93]
Mass spectral profiles	Serum	Cisplatin/ Docetaxel	SELDI-TOF MS	The predictive model was tested in a validation set (n-31). Cross-validation SVM yielded 83.3% sensitivity and 85.7% specificity	[94]
Esophageal Cancer					
Thioredoxin domain-containing protein 4 precursor (↑) **Cystathionine gamma-lyase (↑)**	ESCC Cell lines	Cisplatin	2DE & MALDI-TOF-TOF	Western blot RT-PCR	[95]

Putative biomarker	Source	Resistant drug	Proteomic Method	Validation	Ref.
HSP27 (↓)	Adenocarci-noma tissue	5-Fluorouracil/ Cisplatin	Radioactive 2DE & MS (MALDI-TOF MS & LC/ESI-MS/MS & MALDI-TOF-TOF)	IHC qRT-PCR	[96]
Serum amyloid A (↓) Transthyretin (↑) Apolipoprotein A-I (↑)	Plasma (adenocarci noma xenograft)	Epirubicin Cisplatin or 5-Fluorouracil	SELDI-TOF MS & nanoLC-MS/MS	Biomarkers were validated on clinical samples collected from patients with esophageal cancer prior and after chemotherapy	[97]
Bladder cancer					
Adseverin (↑)	Cell lines	Cisplatin	2DE & nanoLC-MS/MS	Western blot siRNA transfection	[99]
Acute myeloid leukemia (AML)					
Mass spectral profiles	Peripheral blood plasma	Idarubicine + Cytosine Arabinoside	SELDI-TOF MS	Analysis in a validation set (33% of samples set aside)	[100]
Gastric Cancer					
Sorcin (↑)	Cell lines	Vincristine	2DE & MS (MALDI-TOF MS & ESI-Q-TOF-MS)	Westrern blot siRNA transfection	[101]
HSP27 (↑)	Cell lines	Vincristine	2DE & MS (MALDI-TOF MS & ESI-Q-TOF-MS)	Western blot qRT-PCR antisense oligonucleotide	[102]
PK-M2 (↓)	Cell lines	Cisplatin or 5-Fluorouracil	2DE & MALDI MS	Western blot antisense oligonucleotide	[103]
Colorectal Cancer					

Putative biomarker	Source	Resistant drug	Proteomic Method	Validation	Ref.
Mitochondrial F(1)F(0)-ATP synthase (↓)	Cell lines	5-Fluorouracil	2DE & MALDI MS	Western blot siRNA transfection	[105]
Pancreatic Cancer					
HSP27 (↑)	Cell lines	Gemcitabine	2DE & LC-MS/MS	Western blot IHC siRNA transfection	[106]

4. Discussion

As indicated above, an emerging research question in clinical proteomics is the discovery of specific protein markers that would allow the guidance of targeted therapies with definite clinical outcome in cancer patients. The proteomic studies described in this review have attempted to investigate drug resistance in cancers and a large number of putative biomarkers associated with chemoresistance have been identified.

In the majority of the published work presented, quantitative proteomics technologies have been applied to study the differential proteomic profiles in chemoresistant cancer cell lines rather than in clinical samples. Cultured cell lines is an excellent source for protein biomarker discovery since protein samples can be obtained in relative large quantities however, detected protein levels may not always reflect the in vivo clinical state and therefore, careful interpretation of the obtained MS data and further validation in cancer tissues is imperative [107]. On the other hand, proteomic analysis using tumor tissue samples is technically challenging due to tumor heterogeneity and the limited quantity of samples, especially in the case of predictive pre-treatment biopsies [15,27]. Another alternative and highly attractive proteomic approach is to identify circulating biomarkers by analysing body fluids near to the tumor site of origin such as blood, serum and plasma, urine or saliva, which can be obtained simply and less-invasively [108]. However, only a limited number of studies exist in which biological fluids were analysed to discover predictive biomarkers. The proteomic analysis of biofluids is particularly challenging due to the large dynamic ranges of protein expression levels (10^{12} for blood serum) [24]. Tumor-secreted proteins are expected to be detected at very low concentrations [109] and despite methods developed for the efficient depletion of the most highly abundant proteins, the dynamic range still remains large [24].

From all the data presented, it appears that the panel of differentially expressed proteins (DEPs) identified for the same type of cancer varies from one study to another, suggesting a lack of experimental standardization or issues of interpatient and intratumor heterogeneity in the cases of analysed clinical samples [23]. On the other hand, in most comparative studies more than one biomarker have been identified that might contribute to chemoresistance. These findings indicate that drug resistance is a multifactorial phenomenon and thus, instead of targeting any single mechanism, combination therapies targeting multiple mechanisms may be

necessary for enhancing chemosensitivity of cancer [8]. Interestingly, as it can be seen in Table 1, in which all the identified putative biomarkers are summarized, some biomarkers are indicative of drug resistance in more than one type of cancer. In particular, sorcin was found to be up-regulated both in gastric and in NSCLC resistant cancer cell lines, while down-regulation of the pyruvate kinase isoenzyme M2 (PK-M2) was associated with chemoresistance in gastric and ovarian cancer. In addition, HSP27 overexpression has been shown to be associated with resistance to chemotherapy in gastric and pancreatic cancer. More importantly, the role of HSP27 and PK-M2 in drug resistance has also been described in multiple lines of drug resistant cancer cells regardless of the chemotherapeutic regimen and the origin of the cancer cells [8]. Therefore, these proteins might have a great potential as a common target towards sensitizing drug resistance in human cancers. Remarkably though, in the study of Langer et al. [96] high levels of HSP27 in pre-treated adenocarcinoma biopsies obtained from patients with advanced esophageal adenocarcinoma was correlated to chemosensitivity instead or resistance. According to the authors, this contradictory finding could be most likely due to different regulations depending on the tumor (type, stage) and the investigated collectives (e.g. therapeutic regimen) and is one of the few instances in which low HSP27 expression in cancer is indicative of negative treatment outcome.

Most of the biomarkers discussed in this review are very promising and have been adequately retrospectively validated in small selected sample sets by immunoassays and functional studies. However, future implementation of these candidate biomarkers in clinical application requires further research in multicentre prospective screening studies and large clinical trials [23].

5. Conclusion

Whilst still in its infancy, the use of high-throughput, sensitive and robust proteomic technologies is a very powerful tool for the discovery of predictive biomarkers that may pave the way for personalized medicine. If designed prospective clinical trials confirm the preclinical data, predictive biomarkers can be used to guide treatment decisions by selection of the most appropriate therapeutic regimen if active therapies are available [110]. In addition, biomarkers associated with drug resistance may be used as new drug targets for future therapeutic intervention.

To determine specific molecular signatures of sensitive and resistant cell lines and tumor samples to traditional cytotoxic chemotherapy seems a feasible way to detect resistance but clinical validation seems the major obstacle at present. Thus far, no markers for drug response testing have been adopted in clinical practice. Consequently, in the near future, rigorous validation of the new tumor biomarkers in prospective studies is urgently needed for moving forward from the biomarker discovery phase stage to their application in the clinic.

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