Progress in mass spectrometry-based proteomic research of tumor hypoxia (Review)

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Abstract. A hypoxic microenvironment effects various signaling pathways in the human body, including those that are critical for normal physiology and those that support tumorigenesis or cancer progression. A hypoxic tumor microenvironment, in particular, modulates cell migration, invasion and resistance to radiotherapy and chemotherapy. Development of the mass spectrometry (MS) technique has allowed for expansion of proteomic study to a wide variety of fields, with the study of tumor hypoxia being among the latest to enjoy its benefits. In such studies, changes in the proteome of tumor tissue or cells induced by the hypoxic conditions are analyzed. A multitude of hypoxic regulatory proteins have already been identified, increasing our understanding of the mechanisms underlying tumor occurrence and development and representing candidate reference markers for tumor diagnosis and therapy. The present review provides the first summary of the collective studies on tumor microenvironment hypoxia that have been completed using MS-based proteomic techniques, providing a systematic discussion of the benefits and current challenges of the various applications.

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1. Introduction

When blood supply to a solid tumor is limited, such as by interrupted or abnormal vasculature, a hypoxic microenvironment is generated (1). Effects of the hypoxic state, and the induced physiological and tumorigenic changes, have been extensively studied (2-4) and include cell metastasis (5), neovascularization (6), cell invasiveness (7) and resistance to radiotherapy, and chemotherapy (8-10). The various regulatory factors contributing to hypoxia-associated signaling and mechanisms have been proposed as targets for oncology research (11), and extensive proteomic studies of hypoxia in many types of tumor cells have been carried out and reported in recent years.

The concept of 'proteome' was first publicly proposed in 1994, and was described as the full profile of proteins expressed by all genes in the genome of a cell or the particular profile of proteins expressed by a cell at a certain phase (12). Since then, proteomic study has been applied to many fields of biological research (13-15). Development of the mass spectrometry (MS) technique significantly advanced our ability to identify proteins and now represents the main research method of proteomic analysis (16-18). MS-based 'shotgun' proteomics is the prototype method and is currently in wide use (Fig. 1). The mass spectrometer instrument consists of a sample inlet, ion source, mass analyzer, detector and data system (Fig. 1B). After passage through the sample inlet, peptides are ionized in the ion source chamber and separated in the mass analyzer chamber. The mass-to-charge ratio (m/z) of the peptides is determined by the detector. Finally, the identity of the proteins is confirmed by bioinformatic analysis, which frequently involves automated searching of protein databases (Fig. 1C and D).

Protein samples are usually prepared for MS detection by gel electrophoresis separation (or resolution by other methods) and digestion (Fig. 1A). The most widely used gel-dependent protein separation technique is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),

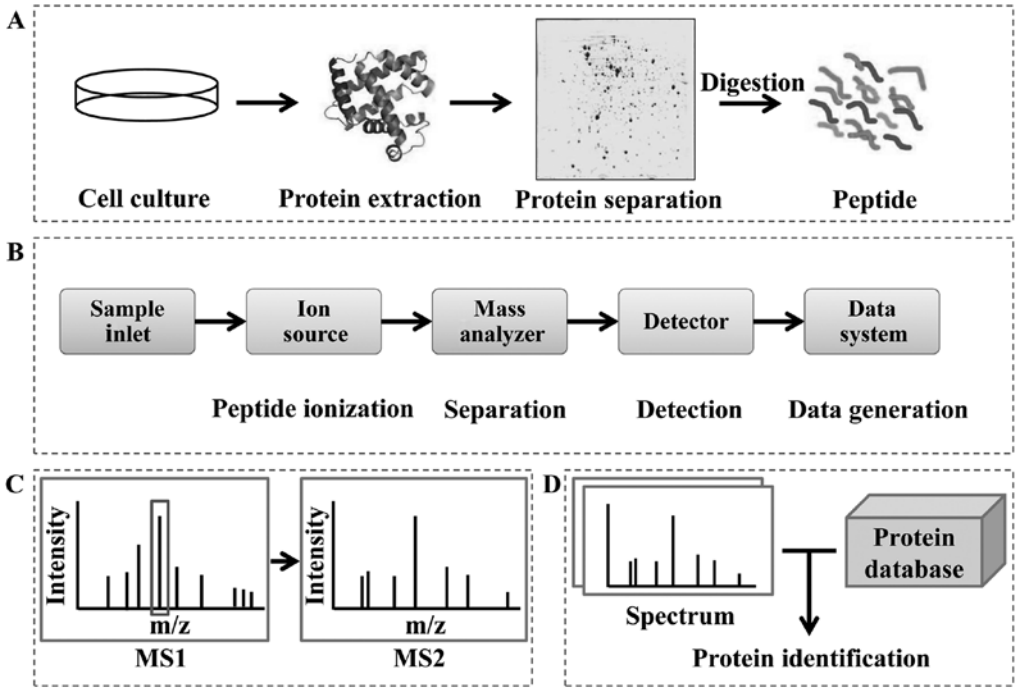

Figure 1. MS-based proteomic analysis. (A) Cells are cultured under experimental conditions and the extracted proteins are usually separated using a gel-dependent technique, such as 1D SDS-PAGE or 2DE, after which the resolved proteins are digested by trypsin and the resultant peptides are subjected to MS for identification. (B and C) Peptides are separated by liquid chromatography and ionized by an ion source upon passage through the instrument's sample inlet, after which they are separated in the MS analyzer. In C, the MS1 plot has been produced upon detection of the peptide m/z and the MS2 plot has been produced by fragmentation of the m/z peptide, showing the requirement of high intensity. (D) Protein identification. The peptides are identified following search of an MS/MS database, leading to identification of the corresponding proteins.

which was established in 1967 by Shapiro *et al* (19,20) and was improved by Weber and Osborn in 1969 (21). SDS, an anion detergent, serves to break the hydrogen bonds between molecules, causing breakdown of the protein structure. Protein migration rates in gel electrophoresis depend on the molecular weight (MW), which itself is affected by the concentration of the gel. Another technique for separating proteins is two-dimensional gel electrophoresis (2DE), which was established in 1975 (22). In this method, proteins are firstly separated by their respective isoelectric points (pIs) and secondly, in the perpendicular direction, by their respective MWs, ultimately distributing throughout the gel as pI/MW-dependent protein spots. On the basis of 2DE, 2D-difference in gel electrophoresis (DIGE) was introduced by Unlü *et al* (23). This expanded method relies on protein labeling with fluorescent dye labeling to improve the accuracy of quantitation (24). Different proteins can then be detected by the MS technique and finally identified by bioinformatics. Since 2DE allows for the properties of proteins to be visualized (i.e. pI, MW and relative abundance), it has emerged as a key tool for comparative proteomic studies. However, the experimental operations for 2DE are not automated, the separated proteins cannot be accurately quantified and the proteins with extreme pIs or insolubility are not separated well by 2DE.

To address the above concerns, quantitative proteomic techniques have been developed, representing two categories segregated by the use of either a stable or unstable isotope for labeling of the protein samples. In label-free quantification, the quantity can be determined based upon the extracted ion current (XIC) (25) or using the method of spectral counting (SC) (26). The usual stable isotopic labeling quantification techniques, however, include the stable isotope labeling by amino acids in cell culture (SILAC) (27), isotope-coded protein labels (28), isobaric tag for relative and absolute quantitation (iTRAQ) (29) and tandem mass tag (30). The same peptides with different labels are presented in the same spectrogram as isotopic peaks with fixed difference in MW. In label-free quantification, sample preparation is simple (no label is required) and the cost is relatively low, but the quantitative efficiency depends on the stability of the mass spectrometer instrument itself. The quantitative proteomic techniques using an unstable (radioactive) isotope label is very specific and provides direct detection, but the well-known safety issues are a disadvantage. In contrast, stable isotope labels usually have high sensitivity and high quantitative accuracy, but the operation is complicated and features a relatively high cost.

Proteomic techniques make it possible to gain greater insight into hypoxia by improving our ability to analyze the underlying molecular mechanisms and consequences of the hypoxic condition through protein expression. Herein, we describe, for the first time, the collective applications of MS-based proteomic analysis for assessing tumorous microenvironment hypoxia. Various types of tumors have been subject to the study of hypoxia effects and a multitude of proteins affected by the hypoxic condition have been identified. Several proteins have been found repeatedly across the studies of different tumor types, suggesting their potential roles in general hypoxic regulation. In addition, proteins have been identified that are distinctive to specific tumor types, and as such represent possible biomarkers. A better understanding

of the hypoxia factors that compose the dynamic tumor proteome may help advance both our overall understanding of tumorigenesis and tumor cell survival, as well as our ability to generate innovative antitumor therapies.

2. 2DE/2D-DIGE-coupled MS

The 2DE technique has been widely used in comparative proteomic analysis. For this, protein samples representing different experimental conditions are separated on respective gels (as detailed above) thus, that the relative expression level, pI or MW changes visualized in one gel can be compared to the other(s) for the different conditions being assessed. The differentially expressed proteins can then be selectively identified by coupled MS detection. The more newly developed 2D-DIGE technique, in contrast, provides more accuracy in finding differentially expressed proteins, due to the fluorescent dyes used to label the various samples. Both of these methods, however, have successfully been applied to the protein samples of various tumor types, including head neck squamous cell carcinoma (HNSCC), cervix carcinoma (i.e. HeLa cells), pancreatic ductal adenocarcinoma (PDAC), leukemia and breast cancer.

HNSCC. Chen *et al* (31) reported a proteomic study of hypoxia-regulated proteins in HNSCC in 2004. In total, more than 1,000 protein spots were separated in gels under hypoxic and normoxic conditions. Twenty of those proteins showed increased expression (>1.5-fold) during hypoxia. Among these, the IκB kinase β (IKKβ) and MKK3b are known to be highly expressed in cancer, and the density-regulated protein 1, $P150^{glued}$, nuclear transport factor 2, binder of ARL 2, paxillin and transcription termination factor I represented newfound hypoxia-inducible factors. Further analysis of IKKβ in hypoxia revealed that it is a regulator of the nuclear factor-κB (NF-κB) transcription factor under hypoxia conditions, which was in accordance with previous studies (32). In patients with HNSCC, this enzyme mediates cell survival during hypoxia and the expression correlates with tumor oxygenation, suggesting that it may represent a novel endogenous tumor marker.

Cervix carcinoma. In 2007, Magagnin *et al* (33) reported on protein expression changes in HeLa cells under hypoxic and reoxygenation conditions. Seven of 14 differentially expressed protein spots for the reoxygenation samples were identified by electrospray ionization (ESI)-QTRAP® MS (AB SCIEX, Framingham, MA, USA) and were found to be associated with cellular processes and stress response pathways. These proteins included VCP/p97, FUSE binding proteins 2 and 3 (FBP2/FBP3) and ribosomal protein P0 (RPP0). The VCP/p97 protein is a hexameric ATPase and it is associated with various processes of cellular regulation (34). The FBP2 and FBP3 proteins are associated with RNA processing and have been demonstrated as being involved in the regulation of protein expression in response to hypoxia (35,36) and other conditions, such as irradiation (37) and oncogenic signaling (38). Overall, these identified proteins play roles in the resistance of tumor cells to hypoxic exposure. Magagnin *et al* (39) further reported on a study of proteome changes in HeLa cells in 2008. Several proteins were found to be regulated by mammalian target of rapamycin (mTOR) and its downstream effector 4E-BP1, both under hypoxic and normoxic conditions. These proteins had been previously reported as associated with tumor cell motility, invasion and metastasis, all of which contribute to the adverse tumor phenotype (40-43).

PDAC. A study of hypoxia-regulated proteins in PDAC was reported by Cui *et al* (44) in 2009. Thirty-five unique expressed proteins were identified in PDAC, as compared to proteins in pancreatic ducts; these included 21 upregulated and 14 downregulated proteins. Seven of the 21 increased proteins had been previously reported to be regulated by hypoxia (45-50), and these included Annexin A5, fatty acid synthase, fructose bisphosphatase aldolase A, glucose-regulated protein 78 (Grp78), macrophage migration inhibitory factor (MIF), phosphoglycerate kinase-1 (PGK-1) and transketolase.

Leukemia. Liao *et al* (51) reported a study of different leukemic cell lines, in which they aimed to identify direct targets of hypoxia-induced factor (HIF)-1 and provide new insights into the molecular mechanisms associated with HIF-1α-mediated tumor metastasis. According to the results, Annexin A1, CapG and S100A4 genes were significantly induced by hypoxia in 5 of the adherent cell lines tested as well as in the non-adherent U937 cells, and the expression could be blocked by silencing the HIF-1α gene. CapG is a member of the gelsolin superfamily and it plays a role in the regulation of actin filament length by severing filaments (52). S100A4 is involved in cancer cell migration and invasion (53,54) and the functions were identified in the present study. Ultimately, S100A4 and CapG have been suggested as novel direct target candidates of HIF-1α.

Breast and colon cancers. Bartkowiak *et al* (55) reported in 2010 their results of a study of the breast cancer disseminated tumor cell (DTC) line, which is established from bone marrow, a known hypoxic microenvironment. The unfolded protein response (UPR) proteins Grp78 and Grp94 and the protein disulfide-isomerase were identified as overexpressed. These UPR proteins could be applicable as novel biomarker proteins for DTC detection in cancer patients. A few years later, in 2013, Grandjean *et al* (56) identified a potential colon cancer biomarker through their research of the human colorectal carcinoma HCT116 and HT29 cell lines, through which the phosphorylated form of translation elongation 2 was verified as a new tumor-associated antigen.

As a traditional proteomic technique, 2DE coupled MS has been broadly applied in proteomic studies. However, the 2DE protein separation step is not high-throughput and the overall operation is complicated. Moreover, the strategy is not highly discriminative, limiting accuracy of identification of low abundance proteins. Quantitative proteomics were developed to surmount these limitations, and their advantages over the 2DE-based methods have been proven over recent years.

3. Quantitative MS

Label-free quantitative MS

A multitude of label-free quantitative proteomic studies have been carried out in recent years. The simple sample preparation process and lack of label make label-free quantitation easy

to perform. However, the accuracy of this method depends greatly on the stability of the mass spectrometer instrument used. To date, tumor cells from gliomas, hepatocellular carcinoma (HCC) and colorectal cancer as well as human umbilical vein endothelial cells (HUVECs) have all been studied by label-free quantitative MS techniques.

Glioma. Gliomas (brain tumors) have a general poor prognosis. This tumor type was studied by the proteomic approach by Yoon *et al* (57) and reported on in 2014. The exosome and soluble fractions were isolated from the U373MG glioma cell line under hypoxic and normoxic conditions and analyzed; a total of 239 proteins were identified. The upregulated proteins were identified based on a label-free quantitative analysis. Vascular endothelial growth factor, stanniocalcins 1 and 2 (STC1 and STC2, respectively), and insulin-like growth factor binding proteins 3 and 6 (IGFBP3 and IGFBP6, respectively) were found to be enriched in the soluble fraction, and lysyl oxidase homolog 2 was found to be enriched in the exosomal fraction. Among these proteins, the STCs and IGFBPs are secretory proteins enriched under hypoxia conditions. Previous studies in the literature have revealed that STC1 is a calcium- and phosphate-regulating hormone in the blood of fish, and the homologues STC1 and STC2 were also subsequently identified in mammals (58,59). The expression level of the STCs correlated with glioma grade in the present study, and they were also found to be related with hypoxia-dependent migration of tumor cells. Thus, the STCs represent potential biomarkers for cancer diagnosis, as was purported by other previous studies (60,61).

HCC. Malignancy progression in hypoxia of HCC was studied by a label-free quantitative proteomic strategy (62). A single significantly increased protein was identified: prohibitin 2 (PHB2). Previous studies have demonstrated PHB2 to be associated with the mitochondria, in various aspects (63-66). When the PHB2 expression was inhibited or the HCC cells were treated with interfering RNAs against PHB2, the cells showed changes in phenotype of adaption ability to hypoxic microenvironment, in resistance to chemotherapy-induced apoptosis and in cell growth. These results suggested that the PHB2 protein plays important roles in HCC malignancy progression, providing a reference for treatments based on this protein.

Colorectal cancer. The HIF-1α gene in HCT116 colorectal cancer cells was analyzed by a label-free quantitative method, with the aim of studying the metabolic pathways in lipogenesis (67). In total, 3,632 proteins were detected and identified. The results revealed that the lipid metabolites are regulated by hypoxia through both HIF1α-dependent and -independent pathways. The HIF1α-dependent pathways include enzymatic steps in fatty acid synthesis and the Kennedy pathway. The HIF1α-independent pathways include palmitate, stearate, PLD3 and PAFC16 pathways.

HUVECs. Considering the necessity of vessel formation in tumor growth, invasion and metastasis, a label-free quantitative proteomic study was conducted using HUVECs under hypoxic conditions. Twenty-seven sialoglycoproteins on the surface of HUVECs were identified as overexpressed (68), in addition to the CD105, neuropilin-1 and CLEC14A proteins that had been previously described by other studies of hypoxia (69-71). Various new proteins were identified as hypoxia regulated, including glucose transporter SLC2A1 (GLUT-1), TMEM16F and stromal cell-derived factor (SDF) 4.

Stable isotope-based quantitative MS

In the SILAC technique, cells are labeled by isotopic amino acid during the culture process. This is an efficient and sensitive method, but the cost of the label and the extensive time required for labeling limit its suitability for all protein samples, particularly those from tissue or plasma. The iTRAQ technique is also effective and is sufficiently applicable to various types of protein samples. Other isotope-based labeling methods, such as those using ^{15}N and ^{18}O, have also been successfully applied in proteomic studies. To date, these collective quantitative MS-based techniques have been used in studies of melanoma, neuroblastoma, mammary cancer, von Hippel-Lindau (VHL) tumor, epidermoid carcinoma, lung cancer and adenocarcinoma.

Melanoma. In 2006, a study of the plasma membrane of the hypoxia-adapted murine B16F10 melanoma cells was reported, in which the quantitative proteomic study was based on $^{16}O/^{18}O$ stable isotopic labeling and multidimensional liquid chromatography-tandem MS (72). In total, 24,853 peptides were identified, corresponding to 2,433 proteins. Under hypoxic conditions, aminopeptidase N (CD13), carbonic anhydrase IX, potassium-transporting ATPase, matrix metalloproteinase 9 and SDF1 were upregulated. Moreover, CD13 and SDF1 were validated in human melanoma cell lines, confirming the increased expression during hypoxia. Identification and characterization of novel hypoxia-induced membrane proteins may almost certainly help in the research efforts aimed at the treatment of melanoma.

Neuroblastoma. Small ubiquitin-like modifier (SUMOP2/3) conjugation, which is usually a consequence of transient cerebral ischemia, was studied using SILAC-based quantitative proteomics in 2012 (73). The neuroblastoma B35 cell line was exposed to the ischemic condition and the changes in levels of specific SUMOylated proteins were assessed using a stable isotope labeling method. Lysates were mixed equally with control proteins and analyzed by 1D SDS-PAGE combined with LC-MS/MS. In total, 118 putative SUMO3-conjugated proteins were identified, of which 22 showed changes in expression that were significant. The protein-conjugated SUMO1/2 and ubiquitin were identified as upregulated and the OGD-induced ubiquitination could be completely blocked by gene silencing of SUMO2/3. Ultimately, the present study demonstrated an association between the SUMO and ubiquitin conjugation pathways.

Mammary cancer. A comprehensive hypoxic quantitative proteomic study was conducted using matrix-assisted laser desorption/ionization MS imaging (commonly known as MALDI-MSI) (74). SILAC-labeled mammary cancer cells were studied and changes in the proteome under hypoxic conditions were identified. At the same time, laser-capture microdissected samples isolated from normoxic and hypoxic regions of mammary tumors were detected by quantitative MS, and the localization of hypoxia-regulated proteins was also analyzed. In the first part of the study, a total of 1,416 proteins were identified, among which 131 had alterations in expression, including 60 that were upregulated and 71 that were downregulated. In the second part of the study, 164 and 118 proteins

were identified in hypoxia and normoxic regions, respectively, and 89 proteins were found to exist in both the hypoxia and normoxic regions.

VHL. The VHL tumor was studied using clear-cell-type renal cell carcinoma (RCC) samples (75). In the present study, the proteomic and phospho-proteomic analyses were combined to analyze 786-O RCC (±VHL) cells in order to identify VHL-associated hypoxic responses. However, the upregulated GLUT-1 (76,77) and N-myc downstream regulated gene 1 (NDRG1) (78), which are known biomarkers of RCC, downregulated intracellular carbonic anhydrase II (CA2) was also identified. CA2 was demonstrated to govern cellular response to the CO_2 fluctuations that accompany hypoxia in tumors.

Epidermoid carcinoma. iTRAQ-based quantitative proteomics have been applied to determine the protein expression alterations driven by hypoxia in various compartments of the A431 human epithelial carcinoma cell line. In 2012, a study of iTRAQ-based quantitative proteomic study of global protein expression and functional changes in A431 carcinoma cells under hypoxic and reoxygenated conditions was published (79). A total of 4,316 proteins were identified, among which more than 1,200 proteins showed changes in their expression. Hypoxia and reoxygenated conditions affected various pathways, including DNA repair, glycolysis, integrin, glycoprotein turnover and STAT1 signaling. Ultimately, the upregulation of glycolysis, integrin, glycoprotein synthesis and downregulation of STAT1 pathways increased metastatic activity of the A431 cells. The non-homologous end-joining pathway (NHEJ) was also identified as upregulated, representing a novel finding at the time. The NHEJ pathway plays an important role in DNA repair of irradiated cells, and its upregulation under the hypoxic condition may enhance the radioresistance of tumor cells (80).

In addition to the total hypoxia-mediated protein profile identified in A431 cells, profiles of the secreted and the exosome proteins, the integrin family of glycoproteins and the chromatin-binding proteins has been the subject of recent investigations using the iTRAQ-based quantitative proteomic strategy. In 2010, Park *et al* (81) reported that the secreted protein profile and exosome profile in A431 cells were beneficial for tumor cells to enhance angiogenic and metastatic potential. Different hypoxic and reoxygenated exposure durations were applied to the tumor cells, and the proteome changes were compared with cells under normoxic condition. The quantitative proteomic analysis revealed that the panel of secreted proteins was involved in angiogenesis, focal adhesion, extracellular matrix-receptor interaction and immune cell recruitment, all of which were consistent with the enhanced angiogenic and metastatic potential behaviors observed under hypoxic or reoxygenated conditions, such as reduced cell adhesion, increased invasiveness and production of a secreted protein panel supporting increased chorioallantoic membrane angiogenic activity. The identified secreted and exosome-associated protein panels suggested that modulation of the microenvironment in order to facilitate angiogenesis and metastasis could be induced in tumor cells by hypoxia.

In 2014, using the same iTRAQ-based quantitative proteomic technique in A431 cells, Ren *et al* (82) reported that the N-glycosylation modifications of integrin α3 (ITGA3) were inhibited by hypoxia and that the hypoxia-induced ITGA3 glycosylation alterations prevented its translocation to the plasma membrane. In this manner, hypoxia-induced decrease of ITGA3 increased the invasive ability of cancer cells. In that same year, Dutta *et al* (83) tested the hypothesis that hypoxia-driven evolution of the chromatome promotes malignant changes and development of therapy resistance in tumor cells; the study used isolated chromatins from A431 tumor cells treated with varying conditions of normoxia, hypoxia and re-oxygenation followed by partial digestion with DNase I. The iTRAQ-based quantitative proteomic analysis of changes in euchromatin- and heterochromatin-associated proteins identified a total of 1,446 proteins, among which 819 proteins were found to have an alteration in the topology under hypoxia. A novel chromatin organizer protein, HP1HP3, was identified that served to mediate chromatin condensation and lead to increased viability, radioresistance and chemoresistance, and self-renewal. Thus, the effects of hypoxia-induced chromatin protein changes on tumor behavior were shown through a proteomic approach and a candidate therapeutic target was identified.

Lung cancer. The effect of multiple stressors on human lung cancer cells has been studied using the proteomic approach (84). In particular, iTRAQ-based quantitative proteomic analysis was applied to lung cancer cells, and bioinformatic analysis revealed that a novel cancer invasion and metastasis-related gene, CIM, was involved in both hypoxic stress- and endoplasmic reticulum stress-associated pathways. These results offer a reference for tolerance against multiple cellular stresses to be evoked by CIM, which plays a role in promoting metastatic cell survival.

Adenocarcinoma. A tumor structure study was carried out with a proteomic approach in 2012 (85). Unlike other studies, the present study divided the solid tumor of interest into four regions: surface (SL), intermediate region (IR), perinecrotic region (PN) and necrotic core (NC). The PN contained non-proliferating hypoxic cells. Protein samples from the four different regions were labeled using the iTRAQ technique and analyzed by quantitative proteomics. In total, 887 proteins were identified, of which 209 were upregulated and 114 were downregulated in PN and NC compared to SL. Moreover, proteins with significant changes in expression were detected in the non-proliferating fraction. The present study offered candidate targets that may be suitable for therapeutic intervention.

4. Advantages and disadvantages of various MS-based proteomics in tumor hypoxia

Diverse MS-based methods have been applied to tumorous hypoxia research, representing three categories of methods: 2DE/2D-DIGE coupled MS, label-free quantitative MS and stable isotope-based quantitative MS (Table I). The 2DE-based MS technique combines 2DE and MS detection, with the identity rate relying on the discriminatory capacity of the gel electrophoresis. Proteins separated by 2DE/2D-DIGE, present in the gel as protein spots, distribute in the relatively same position regardless of which sample is run (i.e. normoxia, hypoxia). Different expression levels can be visually indicated by differences in the size of a protein spot or the grayscale image of the spot in the gel. The proteins of interest can then be cut out and subjected to MS for identification. The MW

Table I. Common proteomic techniques.

Technique	Description	Advantage	Limitation	Application in hypoxia (Refs.)
2DE/2D-DIGE-MS coupled	Proteins are separated by pIs and MWs, distributed throughout the gel as protein spots	Information on abundance, pIs and MWs are presented visually, and differentially expressed proteins are identifiable by the coupled MS	Complex operation that is not automated Proteins with extreme pIs or insolubility are not separated well	(31,33,44,51,55,56)
Label-free quantitative MS	Protein quantity is confirmed by the extracted ion current or spectral counting	Sample treatment is simple (no label is required) and the cost is relatively low Protein information is attained without manipulation (i.e. the labeling)	Quantitative efficiency depends on the stability of the mass spectrometer Repeated experiments are needed	(57,62,67,68)
Stable isotope-based quantitative MS	Proteins are isotope-labeled based on metabolic or chemical technique	High-throughput, high sensitivity and high quantitative accuracy	Cost is relatively high The different labeling techniques have their own limitations	(72-75,79,81-85)

MS, mass spectrometry.

and pI of protein spots can be readily determined since the distribution pattern is according to these two features. However, the disadvantages of this technique are related to these very aspects; the operation itself is complex and time consuming and the reliance on pI and MW for the protein separation leads to poor distinguishment of proteins with extreme pIs or similar MWs.

The label-free quantitative MS technique has been used extensively in more recent years. The XIC-based label-free quantitative technique mainly relies on the m/z and the signal intensity derived from the spectrogram. Changes in the chromatographic peak in each m/z reflect changes in retention time and signal intensity, which reflect the abundance differences that occur in response to the various conditions under evaluation. In practice, however, stability and reliability of an experimental platform needs to be considered, and in this case retention time should be adjusted to ensure alignment for the co-quantitative peptides in different samples. The two most usual methods for alignment are based on characteristic peaks before the database searching is initiated or on identification results after the database searching has been completed.

When assessing the individual quantitative peptides present in each sample, a cross-search algorithm is used to predict the retention time in other samples, which helps to reconfigure the XIC. Quantification results of the corresponding peptides present in each sample can be confirmed by SC-based quantitative technique, which relies on the abundance of peptides generated by protein digestion. A protein with high expression level generates more peptides. In practice, the SC-based method is limited by experimental error during its application in analysis. In general, the XIC-based label-free quantitative technique has more accuracy, sensitivity and repeatability than the SC-based methods, particularly for the identification of low abundance proteins. However, when these two methods are combined a new set of problems arise.

Stable isotopic labeling quantitative proteomic techniques mainly depend on the uses of isotopic label for the protein sample of interest. The same peptides with different labels may present in a single spectrogram as isotopic peaks with fixed mass differences, which may help identify the abundance of these peptides. Both the precursor ion and the fragment ion can be labeled by stable isotope. Precursor ion labeling is classified as either metabolic labeling or chemical labeling. SILAC is a usual metabolic labeling technique, and essential amino acids, such as arginine and lysine (27), labeled with stable isotope are usually used for cell culture experiments. Fragment ion labeling can provide ions with mass differences in MS/MS fragmentation. iTRAQ has emerged as the most widely used method for fragment ion labeling. Four or eight samples can be labeled simultaneously by iTRAQ technique. Fragment ion labeling has advantages in repeatability and convenience for result calculation, which unfortunately can be affected by co-eluted peptides. When the two procedures are compared, the precursor ion labeling has very high efficiency but is very complicated and time consuming.

5. Conclusion and perspectives

Cells in living organisms express particular panels of proteins whose functionality maintains life and provides response to

disease or injury. Similarly, proteins expressed by tumorous cells provide a systematic picture of the cell metabolic activity, represented by the proteome. Proteomic changes under the tumorous hypoxic microenvironment, as compared to the normoxic state, reflect the cell regulation processes involved in tumor development and persistence. MS-based proteomics has been extensively applied in tumor hypoxia studies during the past several years (Table I), allowing for investigation of various tumor cells under hypoxic conditions in order to search for response-related proteins that provide further insights into the underlying molecular mechanisms and identification of candidate biomarkers for clinical use. The more recent introduction of high-throughput MS technologies has facilitated protein detection on a large scale, and a plethora of proteins involved in the hypoxic response have been identified. The inherent differences among these collective studies, such as in their study designs, as well as the internal differences of the tumor cells themselves have resulted in an array of hypoxia-associated proteins, the profiles of which include those related to general hypoxia regulation and those related to specific tumor types.

A series of novel biomarker proteins have also been found by these proteomic studies, including the IKKβ in HNSCC (31), the UPR proteins (Grp78, Grp94 and protein disulfide-isomerase) in DTC (55), and GLUT1 and NDRG1 in RCC (75). The proteins common among the different tumor cell research studies of hypoxic conditions (i.e. likely regulator of the general or less specific, hypoxic response) include the Grp78 protein that was identified in both PDAC (44) and DTC (55), and GLUT-1 that was identified in HUVECs (68) and RCC (75). The overall proteins with differential expression under hypoxic conditions function in a wide array of cancer-related activities with outcome implications, including cell adaptation, migration and invasion, which is in accordance with the radioresistance and chemoresistance effects of hypoxia in tumor cells.

In view of the characteristics of the different methods available for proteomic analysis, the 2DE-based techniques have yielded relatively less proteins than the high-throughput techniques, with the different expression levels being confirmed by comparison of protein spots in gel. As to the MS-based quantitative proteomic studies, the different expression levels have been usually confirmed by bioinformatic analysis of datasets, adding further power to the potential of this approach to identify a greater number of proteins. Nonetheless, the proteins that have been identified to date warrant further research to gain a greater understanding of their functions or relations to metabolic pathways. Future studies, involving other experimental techniques such as metabolomic analysis, should be carried out to keep the progress moving forward on the tumor hypoxia microenvironment.

The 2DE-based separation relies on the pI and MW, which means that proteins with extreme pIs may not be separated well, and other separation methods should be sought out and applied to overcome this limitation. In the functional analyses, it may be important to clarify each of the proteins detailed role in hypoxic regulation. Combination of proteomic studies and other experimental techniques may be suitable to resolve this problem. Moreover, database searching is the most common approach to identify the MS detection data, but the results may be limited by the quality of the protein database used. Proteins which are not annotated in the database may not be identified and other identification methods, such as spectral matching (86-89) and *de novo* sequencing (18,90), should be introduced to the identification process. Along with the development of improved mass spectrometers and bioinformatic algorithms, more and more valuable proteins may be identified by MS-based proteomics in the future, which may offer a better understanding of hypoxia pathways and their potential intervening targets.

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