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Proteomic Study of Esophageal Squamous Cell Carcinoma

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1. Introduction

Comprehensive profiling of genome and transcriptome has identified myriads of alternations at the level of gene and gene expression, which drive malignant development and progression in context of oncology. As a result, qualitative or quantitative changes of protein expression pattern will inevitably ensue during multi-stage of carcinogenesis. In this sense, the proteome is a functional translation of the genome and is the actual manipulator of cellular behavior. Therefore, proteomic profiling of cellular protein constituents should generate the most relevant marker of the functional state of a cell. On the other hand, lack of correlation between mRNA and protein expression have been documented for a variety of genes. Unlike the genome which is static in certain sense, the proteome of a cell is dynamic and changes over time in terms of protein pattern, protein interactions and modifications triggered by external or internal signals[Kolch et al., 2004; Kolch et al., 2005]. Only dynamic information flow through protein circuitry reflects the course of a disease and allows us to track the pathogenetic mechanisms as well as treatment response[Kolch et al., 2005]. Furthermore, examining DNA sequences and measuring mRNA expression do not specify splicing, post-translational modifications, cleavages, protein subcellular localization and complex formations[Banks et al., 2000; Chambers et al., 2000]. There exists a huge information gulf between RNA transcription and protein expression. Proteome represents a much richer source for the functional description of diseases and the biomarker discovery implicated in cancer. Moreover, most of diagnostic assays currently applied in clinical practice are protein-based immunological methods, which are well adapted to standardization and clinical implementation. Proteomic profiling during disease formation and evolution not only provides an integrated understanding of pathogenesis in context of genome and proteome but also holds greater promise to identify the biomarkers of diagnosis and therapeutic targets for diseases such as cancer.

1.1 Epidemiology and etiology of ESCC

Accounting for more than 400,000 deaths per year, esophageal cancer (EC) ranks as the sixth most common cause of cancer-related mortality worldwide[Parkin et al., 2005]. Moreover, about half of world's EC cases newly diagnosed each year occurred in China[Holmes & Vaughan, 2007]. Histologically, esophageal squamous cell carcinoma (ESCC) and esophageal

adenocarcinoma (EAC) contribute to more than 90% of EC[Daly et al., 2000]. In China, ESCC is the predominant histological subtype and account for nearly 90% of all EC[Li et al., 2011]. In developed countries, in contrast, EAC has been increasingly more frequent over the past two decades and has now surpassed the previously more predominant ESCC[Brown et al., 2008; Trivers et al., 2008]. The incidence of ESCC is characterized by its striking geographical distribution across the world. In the extremely high incidence areas, e.g. northern China, the incidence of EC exceeds 100/100 000/year, while the incidence is less than 5/100 000/year in Europe and the USA[Cheng & Day, 1996]. Heavy smoking and alcohol consumption are associated with increased risk of ESCC in developed countries[Brown et al., 2008; Messmann, 2001; Morita et al., 2010], but not major contributing factors in the pathogenesis of ESCC in China, where major risk factors include nutritional deficiency, consumption of pickled vegetables, dietary contamination with nitrosamine or mycotoxin, and low socioeconomic status[Kamangar et al., 2009; Yang et al., 1984]. In light of the poor nutrition status in Linxian, one of the highest incidence areas for ESCC in the world, two large nutrition intervention studies implemented in the late 1980s reported that the combination of selenium/vitamin E/β-carotene significantly reduced total mortality, total cancer mortality and stomach cancer incidence[Blot et al., 1993; Li et al., 1993]. High baseline serum selenium concentrations showed strong protective effects on ESCC and stomach cancer in prospective studies[Mark et al., 2000]. Recently, opposing trends in incidence of EAC and ESCC, i.e. decrease of ESCC incidence and reciprocal increase of EAC incidence has been observed not only worldwide but also in high risk areas in China, pointing to the roles of economic level and lifestyle factors in EC pattern change[Devesa et al., 1998; Fan et al., 2008; Hongo et al., 2009]. In addition, familial aggregation of ESCC has been reported in high-risk areas for ESCC[Chang-Claude et al., 1997]. Taken together, these facts indicate that both genetic susceptibility and environmental risk factors contribute to the etiology of ESCC.

1.2 Current situation of clinical management of ESCC

Early detection of ESCC is formidable and the majority of ESCC patients have advanced metastatic disease at initial diagnosis. Therefore, 40-60% ESCC patients are inappropriate for curative resection, which remains the primary treatment of ESCC as it provides sustained palliation of dysphagia and the best chance of cure[Hagymasi & Tulassay, 2007; Triboulet et al., 2001]. Nonetheless, more than 50% ESCC develop recurrence within 2-3 years after surgery[Dresner & Griffin, 2000; Hulscher et al., 2000; Nakagawa et al., 2004]. Moreover, the overall 5-year survival rate is < 10% despite significant improvements in surgical techniques and adjuvant chemoradiation[Lightdale, 1999]. In contrast, the 5-year survival rate for EC patients at early stages could be as high as 90%[Hu et al., 2001]. Long-term survival correlates with stages of EC, as evidenced by 40-62% of 5-year survival rate for stage I and IIA contrasting with 18-25% for stage IIB and III of EC[Iizuka et al., 1989]. This suggests that the reasons for this disappointingly low survival rate include ineffective screening tools for high-risk population, cancer detection at an advanced stage, high-risk for recurrence, lack of targets for treatment, unreliable noninvasive tools to monitor complete response to chemoradiotherapy and so on. Clearly, identification of effective biomarkers for early detection, monitoring tumor progression and potential therapeutic targets offer the best chances to lower the morbidity and mortality of ESCC.

1.3 Molecular biology studies of ESCC and its contribution to clinical management

Extensive molecular biology studies of ESCC have identified a wealth of dysregulated molecular events involved in esophageal carcinogenesis, which cover a broad range of genes

with diverse functions, such as vulnerable genes to chemicals, tumor-related genes, tumor suppressor genes, metastasis genes, apoptosis gene, proliferation genes, etc[Enzinger & Mayer, 2003; Greenawalt et al., 2007; Kwong, 2005; Lin et al., 2009]. Moreover, epigenetic alterations, chromosomal changes and transcriptional changes have also been found to play crucial roles in the pathogenesis of ESCC[Abnet et al., 2010; Greenawalt et al., 2007; Wang et al., 2010]. Although these findings improve our general understanding about the molecular biology of ESCC, the appropriate biomarkers for high-risk population screening, for clinical diagnosis and prognosis, for evaluation of treatment efficiency have not been identified yet. Therefore, it is imperative to search more effective biomarkers for such purposes.

2. ESCC analysis by proteomics

2.1 Advantages of proteomics compared with genomics

The completion of human genome sequence did not ensure panacea solutions to all problems related to biological deregulation. In fact, human proteome is far more complex and dynamic than genome sequence. It is estimated that the human genome contains about 32 000 protein coding genes, which code for 100 000 to 10 million proteins due to alternative RNA splicing, overlapping of transcription units, post-translational processing and modifications[Lander et al., 2001; Venter et al., 2001]. Thus, a big disparity between genome and proteome exists, which indicates that the combinatorial diversification of regulatory networks lead to functional evolution of proteins. Through detecting the functioning units, proteomic studies generate a protein fingerprint, which reflects both the intrinsic genetic programme of the cell and the impact of its immediate environment. Therefore, proteomics is valuable for biomarker discovery since its application provides higher opportunity to identify genuine determinants or causal factors involved in biological functions or the pathogenesis of disease.

2.2 Two-dimensional electrophoresis-based proteomic findings of ESCC

Two-dimensional electrophoresis (2DE) has been used for over 30 years now due to its high resolution for the separation of complex protein mixtures. In combination with mass spectrometry, 2DE has been so far the most commonly used method for analyzing protein expression and identity. Our laboratory used 2DE to profile the proteome from ESCC tumors and matched adjacent non-cancer mucosa, and proteome from immortalized esophageal cell line and cancer cell lines. Comparative analysis and MS for protein identification showed that the over-expressions of four proteins were common in ESCC tissues and cancer cell lines, which include tropomyosin isoform 4 (TPM4), prohibitin, peroxiredoxin (PRX1) and manganese superoxide dismutase (MnSOD); the expressions of another three proteins, i.e. stratifin, prohibitin, squamous cell carcinoma antigen 1 (SCCA1), were correlated inversely with dedifferentiation of ESCC[Qi et al., 2005; Qi et al., 2008]. Immunohistochemistry (IHC) analysis showed that loss of expressions of annexin A2 and stratifin were 45% and 64% in ESCC, respectively[Qi et al., 2007a; Qi et al., 2007b; Ren et al., 2010]. Differential expressions of ten proteins including TPM1, SCCA1, stratifin, peroxiredoxin 2 isoform a, alpha B-crystalline, annexin A2, heterogeneous nuclear ribonucleoprotein L (hnRNP L), triosephosphate isomerase1 (TPI), laminA/C, and cyclophilin A (CypA) can be observed as well. Our findings may suggest that these differential proteins contribute to the multistage process of carcinogenesis, tumor progression, and invasiveness of ESCC. Published in the same issue, Zhou et al found 28

proteins aberrantly expressed in ESCC cancer cells with at least three-fold difference between ESCC and normal epithelial cells[Zhou et al., 2005]. The overlap between these two studies was quite small. Only expression of SCCA1 was commonly down-expressed in ESCC, but transgelin showed increased expression in tumor in our study and decreased expression in Zhou's study. The disparity of proteins identified between these two studies may be due to different sample source, different methods used by these two groups, such as laser capture microdissection vs. bulk tissues, 2D-DIGE vs. silver staining. Later, five groups reported proteomic signatures associated with ESCC using ESCC samples collected from different regions of China, including high risk areas for ESCC such as Linzhou, Xinjiang and low risk areas like Beijing and Guangdong, but only four reports displayed details of identified proteins. Interestingly, more overlap of the identified proteins came from Fu's study and ours, both of which used ESCC samples from Linzhou, one of the highest areas for ESCC adjacent to Taihang Mountain[Fu et al., 2007]. The commonly identified proteins with the same change direction included alpha enolase, TPM, tubulin, prohibitin and PRX2. Although the prevalence of ESCC in Xinjiang is comparable to Linzhou, the protein signatures were unique to sample origin, indicative of more important roles of environmental, ethnic or hereditary factors in the carcinogenesis of ESCC[Liu et al., 2011]. It seems that hsp27 was a general molecular events involved in ESCC since four out of five studies observed down-expression in ESCC except ours. Only one among seven studies performed survival assay after identifying the candidate proteins by ESCC proteomic profiling. Du et al. reported that over-expression of calreticulin and GRP78 could predicate poor prognosis of ESCC[Du et al., 2007]. Although 2DE is indeed a very useful method for biomarker discovery, more examinations of the biological functions and the clinical relevance of biomarker candidates involved in ESCC are necessary to verify its clinical value.

Two reports described the proteomic signatures of ESCC with samples from Japan. Nishimori et al. used the agarose IEF gel in the first dimension, which not only allows for large-scale quantitative comparisons of protein expression but also is able to resolve high molecular mass proteins larger than 150 kDa[Nishimori et al., 2006]. As a result, a different protein pattern was revealed, including a few protein candidates with MW > 70 kDa. Western blot and IHC verified the different expression of a 195 kDa protein, periplakin, between cancer and adjacent non-cancer tissues. Not only was the expression of periplakin significantly down-regulated in ESCC but also translocation of periplakin was observed, which localized at cell-cell boundaries in normal epithelium and dysplastic precursor lesions, and disappeared from cell boundaries and shifted to cell cytoplasm in early cancers. The other research group from Japan used unsupervised classification to analyze the 2D-DIGE protein spots and procured the protein signatures most relevant to clinical parameters with progression of ESCC[Hatakeyama et al., 2006]. The authors developed the largest protein database relevant to ESCC, which identified 240 proteins with expression level associated with carcinogenesis, histological differentiation and the number of lymph node metastases. A significant overlapping was observed between the proteins identified in ESCC with other different types of tumor. In addition, Jazii et al did proteomic profiling using ESCC samples from Iran, another high incidence area for ESCC like northern China, and identified six over-expressed proteins and six under-expressed proteins associated with ESCC[Jazii et al., 2006]. However, the authors only used RT-PCR to verify the loss of β-tropomyosin in ESCC. The functions of identified proteins associated with the development and progression of ESCC include cytoskeletal/structural organization, transport, chaperon,

oxioreduction, proliferation, glycolysis, cell motility, transcription, signal transduction, suggesting multiple dysregulated pathways involved in ESCC. For better understanding the pathogenesis of ESCC and development of biomarkers, integrated and comprehensive studies on these protein candidates are needed.

An alternative approach to identify novel tumor biomarkers is the assessment of immune response elicited by tumor antigen since the humoral immune response to cancer in humans has been evidenced by the identification of autoantibodies to a variety of intracellular and surface antigens in cancer patients with different types of tumors[Chen et al., 2007; Disis et al., 1997; Hong et al., 2004; Soussi, 2000]. In ESCC, a number of reports have documented the presence of autoantibodies in serum against various proteins, including p53, cytokeratins, myomegalin, TRIM21, peroxiredoxin VI proteins, Hsp70, and CDC25B[Bergqvist et al., 2001; Fujita et al., 2006; Fujita et al., 2008; Liu et al., 2008; Shimada et al., 2007; Shimada et al., 2005; Veale et al., 1988]. The proteomic-based approach to identify panels of tumor antigens and related autoantibodies was introduced by Brichory et al. in 2001, which identified anti-annexin I and II antibodies in sera from patients with lung cancer[Brichory et al., 2001]. There have been four articles published by two research groups, which reported the existence of autoantibodies in sera of ESCC patients. The first report was published by Fujita et al from Japan, who used 2DE to resolve protein extracts from ESCC cell line TE-2 as tumor antigens and then probed the blot with sera of ESCC patients, healthy controls and patients with other cancers[Fujita et al., 2006]. One positive spot was identified as PRX VI by MALDI TOF/TOF MS. The frequency of autoantibody against PRX VI was 50% (15/30) in ESCC, only 6.6% (2/30) in health controls and 3.3% (1/30) in colon cancer. Two years later, the same research group discovered augmented concentration of Hsp70 autoantibody in the serum of ESCC patients, which was significantly higher in ESCC patients than gastric and colon cancer, healthy controls[Fujita et al., 2008]. On the other hand, Liu et al. used ESCC tissue protein extracts and autologous sera to search for autoantibodies in ESCC patients and identified autoantibody CDC25B[Liu et al., 2008]. Furthermore, CDC25B expression was significantly higher in ESCC tissues with positive autoantibody CDC25B and significantly correlated with tumor stage. The sensitivity and specificity of autoantibody CDC25B for ESCC detection was 56.7% and 91%, respectively[Dong et al., 2010]. The autoantibody-driven research is indeed a promising approach for the identification of novel serum biomarkers present in ESCC and for the tumor antigen itself, which may aid the diagnosis of ESCC and development of more effective immunotherapies.

Similar to other cancers, development of multiple drug resistance in ESCC is one of major causes of failure to chemotherapy treatment. Furthermore, recent studies have shown that there exists intrinsic sensitivity and resistance to chemotherapy and/or radiotherapy in malignant cells of ESCC, which may predict clinical outcome of ESCC patients receiving neoadjuvant chemotherapy. Prior stratification of ESCC patients according to reliable biomarkers could not only save patients unnecessary adverse effects of chemotherapeutic agents but also render patients more chance to access to alternative curative treatment options. Therefore, it is imperative to define new diagnostic indicators that can reliably predict response to chemotherapy and radiotherapy in advance. A recent study compared the 2DE gels of parental esophageal cancer cell line EC109 and its resistant sub-cell line EC109/CDDP to determine the different proteins spots and identified 44 proteins with potential contribution to chemotherapy resistance[Wen et al., 2010]. In another study, radioactive 2DE proteomic comparative analysis was performed using protein extracts of biopsies from 34 patients with locally advanced EAC receiving neoadjuvant chemotherapy.

The identified proteins with different expression between responders and non-responders were classified into two major families, cytoskeleton proteins and molecular chaperon proteins. Further validation by IHC and RT-PCR showed that weak expression of HSP27 at protein level and mRNA level were associated with non-response to platin-based chemotherapy[Langer et al., 2008]. As serum represents a rich source for biomarker discovery, proteomic spectra were examined using 27 and 12 serum samples of responders and non-responders, respectively, to preoperative chemoradiotherapy in a training set by surface-enhanced laser desorption and ionization coupled with mass spectrometry analysis. A proteomic classifier comprising four mass peaks, at 7 420, 9 112, 17 123 and 12 867 m/z was identified with 93.3% predicative accuracy in the validation set[Hayashida et al., 2005]. Since chemotherapy resistance is a complex and multi-factorial event, proteomic-based studies enable comprehensive characterization of resistance phenotype of malignant cancers, which may lead to identification of potential distinguishing biomarkers between responders and non-responders and lay foundation for further molecular mechanism studies.

In addition of 2DE gel for proteomic studies, surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) is an alternative proteomic tool to profile the serum or other body fluids and define potential protein pattern with diagnostic potential. By profiling of the serum proteome with SELDI-TOF-MS combined with bioinformatics tools, a number of highly sensitive and specific potential diagnosis markers have been revealed in various types of cancers. Wang et al. used weak cation exchange (WCX2) protein chips and SELDI-TOF-MS to profile 130 symptom-free serum samples collected from high-incidence area of ESCC in northern China, Linzhou, which included 63 subjects with normal esophageal mucosa, 40 subjects with basal cell hyperplasia, 27 subjects with dysplasia and 30 ESCC patients. Biomarker pattern's software identified four protein features at m/z of 9 306.61, 13 765.9, 2 942.15 and 15 953.4, which could distinguish normal esophageal epithelium, basal cell hyperplasia, dysplasia and ESCC with satisfactory diagnostic accuracy[Wang et al., 2006]. Xinjiang is one of the high-incidence areas for ESCC and comprise different ethnic peoples including Han decent. Using CM10 protein chips to capture targets from serum, SELDI-TOF-MS and bioinformatics analysis resulted in identification of six protein peaks (m/z 5667, 5790, 5876, 5979, 6043 and 6102) with diagnostic power with sensitivity and specificity of 91.43% and 88.89%, respectively[Xu et al., 2009]. In the case of ESCC profiled by SELDI-TOF-MS, further purification and identification of discriminatory peaks is necessary for development of simple methods for wider clinical application, and to enhance our understanding of the molecular mechanisms of esophageal carcinogenesis as well.

2.3 SILAC-based proteomic findings of ESCC

Quantitative proteomics is one of the hot research fields in post-genomic era, which has been used extensively in oncology to identify biomarkers with diagnostic and therapeutic potential, thereby avoiding proteins without biological importance. In traditional 2DE, quantitative information of protein spots on 2DE gels is represented by staining intensity. Although 2DE is a versatile tool for visualization of thousands of proteins, detection of post-translational modified isoforms and targeting of protein expression alternations, it has inherent limitations, such as limited resolution of membrane or extreme pI proteins, low sensitivity and throughput, poor reproducibility, etc., which result in only part of proteome uncovered[Ong & Mann, 2005]. In this context, two classes of MS-based quantitative

Protein name	T/N ratio	Functions	References
Annexin A2	↑or ↓		[Du et al., 2007; *Liu et al., 2011*]
Annexin A8	↓		[Nishimori et al., 2006]
Annexin I	↓or↑	Calcium-dependent phospholipid binding calcium ion binding	[Du et al., 2007; Jazii et al., 2006; Liu et al., 2011; Nishimori et al., 2006; Zhou et al., 2005; *Zhu et al., 2010]*
Annexin V	↑		[Du et al., 2007]
Annexin VI	↓		[Nishimori et al., 2006]
Reticulocalbin	↑		[Zhou et al., 2005]
S100 A9	↓		[Zhou et al., 2005]
Syntaxin binding protein	↑	calcium ion binding	[Liu et al., 2011]
Translationally controlled tumor protein	↑		[Zhu et al., 2010]
Zinc finger protein 410	↑	Zink/DNA binding	[Du et al., 2007]
Mutuant hemoglobin beta chain	↓	Heme binding	[Du et al., 2007]
Myoglobin	↓		[Zhu et al., 2010]
TPM-4-ALK fusion oncoprotein type 2	↑	NA	[Du et al., 2007; Jazii et al., 2006]
TPM	↓		[Fu et al., 2007; Liu et al., 2011; Nishimori et al., 2006; Zhou et al., 2005]
TPM2	↓		[Jazii et al., 2006; Nishimori et al., 2006; Zhu et al., 2010]
TPM1	↓		[Qi et al., 2005]
TPM3	↑or ↓		[Fu et al., 2007; *Zhu et al., 2010]*
TPM4	↑		[Qi et al., 2005]
TPM isoform	↑		[Fu et al., 2007; Qi et al., 2005]
Vinculin	↓		[Nishimori et al., 2006]
Capping protein, gelsolin-like	↓	Cytoskeleton constituent	[Fu et al., 2007]
Smooth muscle myosin heavy chain 11 isoform SM1	↓		[Nishimori et al., 2006]
Smooth muscle protein	↓		[Liu et al., 2011]
Apha-actinin 4	↑		[Fu et al., 2007]
Tubulin alpha-6, ubiquitous	↑		[Fu et al., 2007]
Tubulin beta-5 chain	↑		[Fu et al., 2007; Qi et al., 2005]
Gamma-actin	↑		[Qi et al., 2005]
Beta-actin	↑		[Fu et al., 2007]
ACTB protein	↑		[Liu et al., 2011]
Profilin-1	↑		[Zhu et al., 2010]
Periplakin	↓		[Nishimori et al., 2006]
Calreticulin	↑	Stress response and immunity	[Du et al., 2007; Jazii et al., 2006]
Calreticulin precursor	↑		[Nishimori et al., 2006]
Keratin 6A	↑	Intermediate filament	[Du et al., 2007]
Keratin 1	↑		[Jazii et al., 2006; Zhou et al., 2005]
Keratin 6	↑		[Nishimori et al., 2006]
Keratin 8	↓		[Zhou et al., 2005]
Keratin 13	↓	Intermediate filament	[Nishimori et al., 2006; Zhou et al., 2005]
Desmin	↓		[Nishimori et al., 2006; Zhou et al., 2005]
Vimentin	↑		[Nishimori et al., 2006]
MnSOD	↑	Superoxide dismutase activity	[Du et al., 2007; Qi et al., 2005]
Proliferation cell nuclear antigen (PCNA)	↑	DNA polymerase activity	[Du et al., 2007; Zhou et al., 2005]
PRO1708	↑		[Du et al., 2007]
Dank-type molecular chaperone HSPAIL	↑ or ↓	Stress response and chaperone binding	[Du et al., 2007; *Nishimori et al., 2006*]
DnaJ(Hsp40) homolog	↑		[Nishimori et al., 2006]

Protein name	T/N ratio	Functions	References
Heat shock protein 27 kDa	↓ or ↑		[Du et al., 2007; Fu et al., 2007; *Liu et al., 2011*; Zhou et al., 2005]
Similar to heat shock congnate 71-kDa protein	↑		[Du et al., 2007]
Heat shock 70kDa protein 8	↓		[Nishimori et al., 2006]
Heat shock protein 70 kDa	↑		[Jazii et al., 2006]
gp96	↑		[Zhou et al., 2005]
GRP78	↑		[Du et al., 2007]
Alpha-B-Crystalline	↓		[Qi et al., 2005; Zhu et al., 2010]
Fibrin beta	↓		[Liu et al., 2011]
Crystal structure of huma recombinant procathepsin B	↑	NA	[Du et al., 2007]
M2-type pyruvate kinase	↑or ↑		[Du et al., 2007; Fu et al., 2007; Liu et al., 2011]
Mutant beta-actin(Q6F5I1)	↑		[Du et al., 2007]
Phosphoglycerate kinase 1	↑		[Du et al., 2007; Nishimori et al., 2006]
Alpha enolase	↑	Energy metabolism	[Du et al., 2007; Fu et al., 2007; Nishimori et al., 2006; Qi et al., 2005]
Beat-enolase	↑		[Fu et al., 2007]
Triosephosphate isomerase	↑		[Zhu et al., 2010]
GAPDH	↑		[Qi et al., 2005]
Aldolase A	↓		[Nishimori et al., 2006]
Fructose-bisphosphate aldolase A	↓		[Zhu et al., 2010]
RNA binding motif protein 8A	↑	mRNA/nucleotide/protei n binding	[Zhou et al., 2005]
Translation initiation factor Eif-1A	↑	Translation	[Zhou et al., 2005]
Transmembrane protein 4	↑		[Zhou et al., 2005]
Transgelin	↓or ↑	Protein binding	[Liu et al., 2011; Qi et al., 2005; Zhou et al., 2005; Zhu et al., 2010]
COMT protein	↑		[Liu et al., 2011]
Early endosome antigen 1	↓		[Liu et al., 2011]
Crystal structure of recombinant human fibrinogen fragment	↑	Protein binding	[Nishimori et al., 2006]
Similar to ubiquitin -conjugating enzyme E2 variant 1 isoform	↓		[Du et al., 2007]
Ubiquitin C-terminal esterase	↑	Protein degradation	[Zhou et al., 2005]
Ubiquinol-cytochrome C reductase complex core protein2	↑		[Nishimori et al., 2006]
Proteosome	↑		[Liu et al., 2011]
Galectin-7	↓	Interactionof cells and cell-matrix	[Zhou et al., 2005; Zhu et al., 2010]
Fatty acid-binding protein	↓	Lipid metabolism	[Zhou et al., 2005]
TGase	↓	Protein modification	[Zhou et al., 2005]
Fascin	↑	actin cross-lining	[Zhou et al., 2005]
SCCA1	↓	Cysteine proteinase inhibitor	[Qi et al., 2005; Zhou et al., 2005]
Proteinase inhibitor, Clade B	↓	Neutrophil elastase inhibitor	[Zhou et al., 2005]
Thioredoxin perosidase	↑		[Zhou et al., 2005; Zhu et al., 2010]
Peroxiredoxin 1	↑or ↓	Redox homeostasis	[*Fu et al., 2007*; Qi et al., 2005]
Peroxiredoxin 2	↓		[Jazii et al., 2006; Qi et al., 2005]
ARK family 1	↑	Carcinogen metabolism	[Zhou et al., 2005]
GST M_2	↑	glutathione transferase activity	[Zhou et al., 2005]
Proteasome subunit βtype 4	↑		[Zhou et al., 2005]
Proteasome subunit βtype 9	↓	Protein degradation	[Zhou et al., 2005]
Prosomal protein p30-33k	↑		[Zhou et al., 2005]
Elongation factor Tu	↑	Translation	[Qi et al., 2005]
(NADP) cytoplasmic	↑	NAD binding	[Qi et al., 2005]

Protein name	T/N ratio	Functions	References
Prohibitin	↑or ↓	Transcription regulation	[*Fu et al., 2007*; Qi et al., 2005]
Neuronal protein	↑	Neuronal growth	[Qi et al., 2005]
Nuclear autoantigenic sperm protein isoform 1	↑	Hsp90 protein binding	[Nishimori et al., 2006]
Myosin heavy chain nonmuscle form A	↓	Actin binding or	[Nishimori et al., 2006]
Caldesmon 1 isoform 1	↓	calmodulin binding	[Nishimori et al., 2006]
Myosin regulatory light chain 2	↓	Ventricular/cardiac muscle isoform	[Jazii et al., 2006; Zhu et al., 2010]
Myosin light chain 2	↓	Regulatory light chain of	[Jazii et al., 2006]
Myosin light chain 1	↓	myosin	[Zhu et al., 2010]
Heterogeneous nuclear ribonucleoprotein A2/B1:B1	↑	RNA binding and processing	[Nishimori et al., 2006]
Heterogeneous nuclear ribonucleoprotein A2/B1:A2	↑		[Nishimori et al., 2006]
Myosin light chain 3	↓	Regulatory light chain	[Zhu et al., 2010]
Myosin light polypeptide 6	↑		[Jazii et al., 2006]
Myosin light chain 6B	↓	Regulatory light chain	[Zhu et al., 2010]
Similar to alpha-fetoprotein	↓	NA	[Nishimori et al., 2006]
Trnasferrin	↓	ferric iron binding	[Nishimori et al., 2006]
Alpha-1-antitrypsin precursor	↓	Proteinase inhibitor	[Nishimori et al., 2006]
Alpha-1-antitrypsin	↑		[Fu et al., 2007]
Procollagen-proline	↓	Oxidoreductase activity	[Nishimori et al., 2006]
Calponin 1, basic	↓	actin binding ; calmodulin binding	[Nishimori et al., 2006]
DNA directed RNA polymerase B (ropB)	↑	Transcription	[Jazii et al., 2006]
GH16431P	↑	NA	[Jazii et al., 2006]
OPTN protein	↓	Protein C-terminus binding	[Fu et al., 2007]
67 kDa laminin receptor	↑		[Fu et al., 2007]
TNF receptor associated factor 7	↑	Signal transduction	[Liu et al., 2011]
Stratifin	↓		[Du et al., 2007; Qi et al., 2005]
Cathepsin D	↑	Aspartyl proteinase activity	[Liu et al., 2011]
Chromosome1 open reading frame 8	↑	NA	[Liu et al., 2011]
Cdc42	↑	GTPase activator activity	[Liu et al., 2011]
LLDBP	↑	NA	[Liu et al., 2011]
Adenylate kinase 1	↓	Adenylate kinase activity	[Liu et al., 2011]
General transcription factor IIH	↓	Transcription	[Liu et al., 2011]
Serpin B5 precursor	↑	serine proteinase inhibitor	[Zhu et al., 2010]
Serpin B3	↑		[Zhu et al., 2010]
Transthyretin [Precursor]	↑	Thyroid hormone-binding protein	[Zhu et al., 2010]
Apolipoprotein A-I [Precursor]	↑	lipid metabolism	[Zhu et al., 2010]
Peptidyl-prolyl cis-trans isomerase A	↑	Peptidyl-prolyl cis-trans isomerase activity	[Zhu et al., 2010]
Cystatin-B	↑	Cysteine-type endopeptidase inhibitor activity	[Zhu et al., 2010]
Serum amyloid P-component [Precursor]	↓	Protein binding	[Zhu et al., 2010]
Phosphatidylethanolamine-binding protein1	↓	Serine-type endopeptidase inhibitor	[Zhu et al., 2010]
Carbonic anhydrase 1	↓	Carbonate dehydratase	[Zhu et al., 2010]
Carbonic anhydrase 3	↓	activity	[Zhu et al., 2010]
Creatine kinase M-type	↓	Creatine kinase activity	[Zhu et al., 2010]

Table 1. Reported differential proteins in esophageal cancer tissues

proteomics methods have been developed, which include extracted ion current (XIC)-based label-free quantification and stable isotope labeling quantification. Stable isotope labeling by amino acids in cell culture (SILAC) is an in vivo metabolic labeling method in which stable isotope-labeled amino acids (Heavy vs. Light amino acids) replace the natural amino acids of preexisting proteome[Ong & Mann, 2006]. We used SILAC medium to label immortalized cells (NE3 and NE6) with heavy stable isotope [U-$^{13}C_6$]-H-Lysine and [U-$^{13}C_6$]-H-Arginine and cancer cells (EC1, EC109, EC9706) with light stable isotope [$^{12}C_6$]-L-Lysine and [$^{12}C_6$]-L-Arginine, respectively. After complete labeling of the cellular proteome, equal quantity of proteins from immortalized cells and cancer cells were mixed and then subjected to SDS-PAGE separation, in-gel trypsin digestion and high performance liquid chromatography on-line with electrospray ionization-MS/MS analysis (HPLC-ESI-MS/MS). Forty-seven candidate proteins with differential expression were identified with our arbitrary criteria, which contains ratio change > 1.5 folds, ≥ 2 peptides for quantification and coefficient of variation < 50%. Then, we characterized the cellular protein expression pattern and secretome derived from cisplatin-resistant sub-cell line EC9706 and its parental sensitive cell line EC9706. By SILAC labeling and MS-based quantification, we successfully identified 74 proteins of cellular origin and 57 proteins of secretome with altered expression levels. Similar to our approach, Kashyap et al. used a SILAC-based quantitative proteomic approach to compare the secretome of ESCC cells with that of non-neoplastic esophageal squamous epithelial cells and identified 120 up-regulated proteins with >2-fold difference in the ESCC secretome[Kashyap et al., 2010]. In addition of previously known increased ESCC biomarkers, i.e. matrix metalloproteinase 1, transferrin receptor, and transforming growth factor beta-induced 68 kDa, a number of novel proteins showed distinct expression pattern, among which protein disulfide isomerase family a member 3 (PDIA3), GDP dissociation inhibitor 2 (GDI2), and lectin galactoside binding soluble 3 binding protein (LGALS3BP) were further validated by immunoblot analysis and immunohistochemical labeling using tissue microarrays. These identified proteins participate in multiple biological functions, including molecular chaperones, cytoskeletal proteins, and members of protein inhibitors family, reducing protein, etc., suggesting multiple dysregulated pathways involving in ESCC.

2.4 Clinical relevance of potential protein biomarkers in ESCC

To answer clinical questions, the protein biomarkers identified by proteomic techniques with potential diagnosis and therapeutic targets for ESCC need to be translated into clinical scenario, which is realized by using clinical samples, such as biopsy samples, resected tissue samples, plasma or serum samples, urine samples, saliva samples, etc. The methods used for validation generally comprise Western blot, IHC and ELISA at protein level, and RT-PCR at transcription level. Using 2DE- and SILAC-based quantitative proteomic approaches, we have identified a total of 78 non-redundant proteins with aberrant expression associated with ESCC, suggesting that these proteins may play functional roles in carcinogenesis of ESCC and may have clinical values. Afterwards, Western blot analysis verified the decreased expressions of three proteins, i.e. SCCA1, TPM1 and αB-Cryst in cancer, in accordance with 2DE quantitative results. At transcription level, SCCA1 mRNA was down-regulated in tumor as well. More importantly, the expression of SCCA1 decreased step by step as a function of precancer lesions progression, which suggests that SCCA1 may take part in the multi-stage transformation of ESCC, even in the earliest stages[Qi et al., 2005]. In the 2DE-based comparative proteomic study using immortalized and cancer cell model, we

Accession no.	Protein name	MW/PI	Scores	Ratio (T/N)	Matched peptides	Functions
TPM3 HUMAN	Tropomyosin alpha-3 chain	32.80/4.53	330.06	0.47	2	Actin binding
TPM4 HUMAN	Tropomyosin alpha-4 chain	28.50/4.52	199.64	0.37	2	
K2C8 HUMAN	Keratin, type II cytoskeletal 8	53.67/5.38	907.48	0.51	4	
FSCN1 HUMAN	Fascin	54.50/7.02	296.56	0.45	2	
LEG1 HUMAN	Galectin-1	14.71/5.18	424.98	0.49	3	Signal transduction
CLIC1 HUMAN	Chloride channel ABP	26.91/4.94	447.94	0.63	4	
1433E HUMAN	14-3-3 protein epsilon	29.16/4.48	400.71	0.66	3	
PRDX1 HUMAN	Peroxiredoxin-1	22.10/9.22	689.77	0.55	7	
PRDX2 HUMAN	Peroxiredoxin-2	21.88/5.59	238.11	0.65	5	
PRDX4 HUMAN	Peroxiredoxin-4	30.52/5.85	367.60	0.34	2	Redox homeostasis
PRDX5 HUMAN	Peroxiredoxin-5	22.01/9.93	522.84	0.60	2	
CBR1 HUMAN	Carbonyl reductase [NADPH]1	30.36/9.53	467.30	0.59	2	
KCRB HUMAN	Creatine kinase B-type	42.62/5.25	711.33	1.67	4	
GSTP1 HUMAN	Glutathione S-transferase P	23.34/5.32	1140.8	0.45	6	
GDIB HUMAN	Rab GDI beat	50.63/6.08	614.67	0.47	2	Metabolic process
DHSA HUMAN	Favoprotein subunit complex II	72.65/7.31	207.55	0.5	2	
ACBP HUMAN	Acyl-CoA-binding protein	10.04/6.16	135.03	0.64	2	
PHS HUMAN	PHS 2	11.99/6.33	170.64	0.43	3	
RL27A HUMAN	60S ribosomal protein L27a	16.55/11.78	233.25	0.59	2	Translation
RSSA HUMAN	40S ribosomal protein SA	32.83/4.64	298.67	0.58	2	
IF4G1_HUMAN	eIF-4-gamma 1	175.4/5.1	650.5	2.15	14	
NPM HUMAN	Nucleophosmin	32.55/4.49	444.46	0.52	2	DNA binding
GRP78 HUMAN	GRP78	72.29/4.92	1869.09	0.50	14	Chaperone binding
CH10 HUMAN	Hsp 10	10.92/9.44	219.29	0.40	3	
G6PI HUMAN	Glucose-6-phosphate isomerase	63.11/9.10	510.30	0.48	5	
UGDH HUMAN	UDP-glucose 6-dehydrogenase	54.99/6.89	604.20	0.53	2	
PPIA HUMAN	Peptidyl-prolyl isomerase A	18.00/9.05	770.25	0.59	9	
ALDOA HUMAN	Fructose-bisphosphate aldolase A	39.40/9.18	386.91	0.59	2	Energy metabolism
PGK1 HUMAN	Phosphoglycerate kinase 1	44.59/9.22	1020.8	0.50	6	
G3P HUMAN	GAPDH	36.03/9.26	1127.9	0.52	8	
IPYR HUMAN	Inorganic pyrophosphatase	32.64/5.47	485.51	0.45	3	
ENOA HUMAN	Alpha-enolase	47.14/7.71	1998.1	0.55	15	
CYTB HUMAN	Cystatin-B	11.13/7.85	144.98	0.43	2	
CPSM HUMAN	Carbamoyl-phosphate synthase 1	164.83/6.30	3115.1	0.24	6	
PHB2 HUMAN	Prohibitin-2	33.28/10.21	546.79	0.47	2	Transcription regulation
CAND1_HUMAN	TBP-interacting protein 120A	136.3/5.4	617.2	1.8	15	
PSME2 HUMAN	Proteasome activator complex subunit2	27.34/5.33	367.19	0.48	2	Cell cycle
MCM7_HUMAN	DNA replication licensing factor MCM7	81.3/6.1	510.8	1.97	13	

Accession no.	Protein name	MW/PI	Scores	Ratio (T/N)	Matched peptides	Functions
ACADV HUMAN	VLCAD	70.35/9.63	841.39	0.35	2	Lipid metabolism
ATPA HUMAN	ATP5A1	59.71/9.61	963.07	0.47	5	
THIL HUMAN	Acetoacetyl-CoA thiolase	45.17/9.63	330.39	0.45	2	
MIF HUMAN	Macrophage migration inhibitory factor	12.47/9.12	267.01	0.61	3	Cytokine activity
ATPB HUMAN	ATPB-3	56.52/5.14	1704.2	0.40	5	Ion transport
VDAC1 HUMAN	VDAC-1	30.75/9.22	548.36	2.32	2	Anion transport
VPS35_HUMAN	hVPS35	91.6/5.2	602.6	1.67	12	Protein transport
HYOU1 HUMAN	Hypoxia up-regulated protein 1	111.27/5.02	1206.8	0.56	2	ATP binding
SMD3 HUMAN	Small nuclear ribonucleoprotein 3	13.91/11.07	330.93	0.49	2	mRNA processing

Table 2. Differential proteins between immortalized and cancer cell lines derived from ESCC identified by SILAC-based proteomics

selected Annexin A2 for validation by Western blot and IHC. Stepwise decrease in annexin A2 protein expression was observed when epithelial cell was transformed malignantly. In poorly-differentiated squamous carcinoma, 46% (5/11) of cancer tissue sample lost annexin A2 protein and 36% (4/11) expressed at weak intensity[Qi et al., 2007b]. In a separate study, IHC was used to determine 14-3-3σ in 60 cases of ESCC, nearby matched normal esophageal epithelium and a variety of ESCC precursor lesions. High level of 14-3-3σ expression was found ubiquitously in normal esophageal epithelium with an immuonstaining score of 8.22 in expression. Protein 14-3-3σ was down-regulated stepwise during the multi-stage development of ESCC. Sixty-four percent of poorly-differentiated squamous cancer lost 14-3-3σ expression with a score of 0.45[Qi et al., 2007a]. In agreement with our results, Ren et al. documented that the level of 14-3-3σ in terms of mRNA and protein was markedly down-regulated in ESCC compared with nearby matched non-cancer tissues. Furthermore, decrease of 14-3-3σ expression was correlated with tumor infiltration depth, lymph node metastasis, distant metastasis and lymphovascular invasion and shorter 5-year survival rate[Ren et al., 2010]. Among the different proteins identified by SILAC-based quantitative analysis using immortal cell and cancer cell model, the clinical values of MIF in tumorigenesis of ESCC was determined as well. Not only the increased expression of MIF was detected in cellular protein but also in the conditioned medium of esophageal cancer cell lines EC1, EC109 and EC9706 compared with immortal cell lines NE3 and NE6. Low frequency and very weak expression of MIF was detected predominantly in basal cells in normal esophageal epithelium, with an immunostaining score of 1.13. Pronouncedly up-regulated expression of MIF occurred in severe dysplasia compared with weak immunostaining in mild and moderate dysplasia. In ESCC, high frequency of intense expression of MIF was observed with a score of 5.46. Furthermore, high expression of MIF was significantly correlated with advanced clinical stages. ELISA tests revealed that there was an increase trend in serum level of MIF in clinically advanced stage IV compared to stage I-III. Functional studies on MIF indicated that MIF knockdown resulted in decrease in proliferation, clonogenicity, non-adherent growth and invasive potential. Our findings indicate that MIF may play crucial roles in malignant transformation of pathogenesis of EC and MIF could become a potential biomarker for high-risk population screening, assessment

of therapeutic efficiency, prognostic evaluation, and molecular targets of developing novel therapeutic regimen as well. In addition of our proteomic results in ESCC, several other reports have looked at the clinical value of potential biomarkers, including cytokeratin 14, Annexin I, SCCA1/2, calgulanulin B and HSP 60, alpha-actinin 4 and 67 kDa laminin receptor, cathepsin D and PKM2, periplakin, calreticulin and GRP78, galectin-7, anti-CD25B antibody[Dong et al., 2010; Du et al., 2007; Fu et al., 2007; Hatakeyama et al., 2006; Liu et al., 2011; Nishimori et al., 2006; Zhu et al., 2010]. Nevertheless, further extensive studies are still necessary to determine the clinical utility of the identified proteins in tumorigenesis and progression of ESCC.

3. Conclusions

Nowadays, the dilemma for cancer control and management is not due to lack of efficient treatment options but diagnosis at late stages. In the case of ESCC in China, five-year survival rate for early stage tumor reaches around 90%[Hu et al., 2001]. Obviously, to detect tumor as early as possible is the key for reducing the mortality and morbidity of ESCC. It is believed that development of ESCC from normal esophageal epithelium takes at least about 10 years, during which diseased epithelium manifests as basal cell hyperproliferation, dysplasia, carcinoma in situ in terms of morphology and finally evolves to malignant neoplasms. As such, carcinogenesis of ESCC is a multi-stage and dynamic process which accumulates ongoing changes at the level of both gene and protein expression.

Proteomic studies from various research groups worldwide have identified distinct dysregulated protein expression pattern associated with ESCC. The discrepancy might reflect the different etiology, different stages of disease and diverse pathways involved, which makes identification of biomarkers for ESCC difficult. In light of a wealth of potential biomarkers associated with ESCC identified so far in the exploratory phase, future large-scale validation studies involving symptom-free patients with precursor lesions in high-incidence area and ESCC patients compared with controls are essential toward clinical application. Therefore, ultimate translation from laboratory into bedside for ESCC biomarkers will require close collaboration and cooperation between researchers and clinicians to look into the clinical utility in diagnosis at early stage, prognosis and monitoring treatment efficiency for ESCC.

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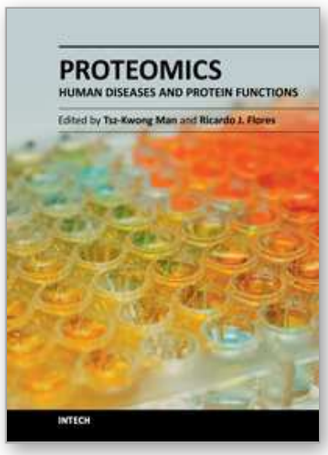

Proteomics - Human Diseases and Protein Functions

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Biomedical research has entered a new era of characterizing a disease or a protein on a global scale. In the post-genomic era, Proteomics now plays an increasingly important role in dissecting molecular functions of proteins and discovering biomarkers in human diseases. Mass spectrometry, two-dimensional gel electrophoresis, and high-density antibody and protein arrays are some of the most commonly used methods in the Proteomics field. This book covers four important and diverse areas of current proteomic research: Proteomic Discovery of Disease Biomarkers, Proteomic Analysis of Protein Functions, Proteomic Approaches to Dissecting Disease Processes, and Organelles and Secretome Proteomics. We believe that clinicians, students and laboratory researchers who are interested in Proteomics and its applications in the biomedical field will find this book useful and enlightening. The use of proteomic methods in studying proteins in various human diseases has become an essential part of biomedical research.

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