

Proteomics and Esophageal Cancer

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1. Introduction

Following to completion of human genome project and accomplishment of the entire human genome sequence, it rose hopes that cure to many diseases would soon come true. This encouraged focusing efforts on the effect of gene expression and the mechanisms by which it could affect medicine, among which cancer. However, searches for the genes within genome (genome: the entire genes of an organism) whose alteration could be the cause of cancer has also been subject of hamper and complications by different mechanisms that genes might be transcribed (transcriptome: the entire transcripts of an organism or organelles within a specific condition) and subsequently into a variety of functional or structural unite known as proteins which can by themselves undergo essential changes [1]. As a powerful approach proteomics entails analysis of gene expression at protein (translation) and protein related levels such as posttranslational modifications, which complement the nucleic acid based level of gene expression. Protein based gene expression analysis is done by analyzing the 'proteome'; the entire protein expressed by a genome in cells, their sub-cellular structures such as organelles and tissues at a given time and specific condition. As a result, proteome is subject of change with time and condition of the being although it is direct product of a genome [2].

The definition of proteomics has changed greatly over the time. While currently it denotes any type of technology that focuses analysis of proteins constituent ranging from a single protein to thousands in one experiment, however it was originally attributed to the large scale protein analysis, high- throughput separation, and subsequent identification of proteins resolved by 2-dimensional polyacrylamide gel electrophoresis (2DE). 2DE is still the method of choice for protein separation and identification [2]. In subsequent sections, we provided a brief description of 2DE, proteomics, and its application in cancer research, the proteins and molecular markers, which were identified in esophageal cancer using this methodology.

2. Two Dimensional Electrophoresis (2DE) and protein identification

The first successful two-dimensional electrophoresis dates to the early 1970s by coupling denaturing IEF (isoelectric focusing) with the SDS-PAGE. Due to awkward process of 2DE, it was relatively unpublicized in its early advent; however, the story has substantially reversed several years later when the astonishing paper that revolutionized application of 2DE was published by O'Farrell [3]. By developing technical aspects of 2DE, O'Farrell was able to resolve hundreds of polypeptides in a single gel and in the same experiment. Since then, analysis of complex protein entities by 2DE has significantly improved, as in the late 1980s, 2DE has reached to a fully developed technique [4]. Though there is always space for development, 2DE still is subject of ongoing advancements along with seeking alternative methods for combining or replacing 2DE in order to achieve higher protein resolution. Nevertheless, the main argument that has put forth application of 2DE is that at present other methods are no more powerful as 2DE is or are hard enough to handle protein complement of the entire genome.

2DE is composed of proteins or polypeptides separation in two orthogonally (right angle) dimension techniques such that in one dimension separation is done based on isoelectric pH point (pI) of protein (or polypeptides) by a process which is called isoelectric focusing (IEF) and the second dimension based on their molecular weight. As a result of IEF proteins are separated based on their charge. Subsequent to IEF, proteins are further resolved or separated in the second dimension based on their molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

From its early advent a requirement for 2DE was separation and comparison of complex protein mixtures with high resolution and reproducibility. The development of immobilized pH gradients (IPGs) on strips for IEF has fundamentally improved and solved this requirement, allowing intra- and inter-laboratory comparison of the separated protein profiles possible. The separated proteins can be detected by staining with dyes or metal ions. Silver staining is the most commonly used method for detection of proteins. The method is 100 folds more sensitive than other dye based staining methods such as Coomassie blue, however radiolabeling is still the most sensitive method, which can be used for autoradiography or fluorography of proteins (figure1).

Following to separation of proteins it is required to identify them in downstream process. While different methods of protein identification were established during past decades, nonetheless, peptide mass fingerprinting in combination with mass spectrometry based methods such as MALDI/TOF, MALDI/TOF/TOF, LC/MS/MS mass spectrometry as well as other methods which all are based on the mass of amino acids and peptides are commonly used for protein identification.

In addition; nowadays 2DE databases are available and could be used as a replacement or as a mean of preliminary analysis of the experimentally obtained 2DE separated and scanned polypeptides against such 2DE protein profile provided by databases for a specific cell type, organelle, body fluids, or tissues. While such supplementary sources of information are useful for primary analysis, however mass spectrometry based methods are still the best mean of protein identification with high confidence. The complexity and quantity of data available from 2DE gel patterns can be handled by image analysis techniques using automated

computer analysis systems, which can provide both qualitative and quantitative information for polypeptides resolved in an individual gel and provide pattern matching between gels [2].

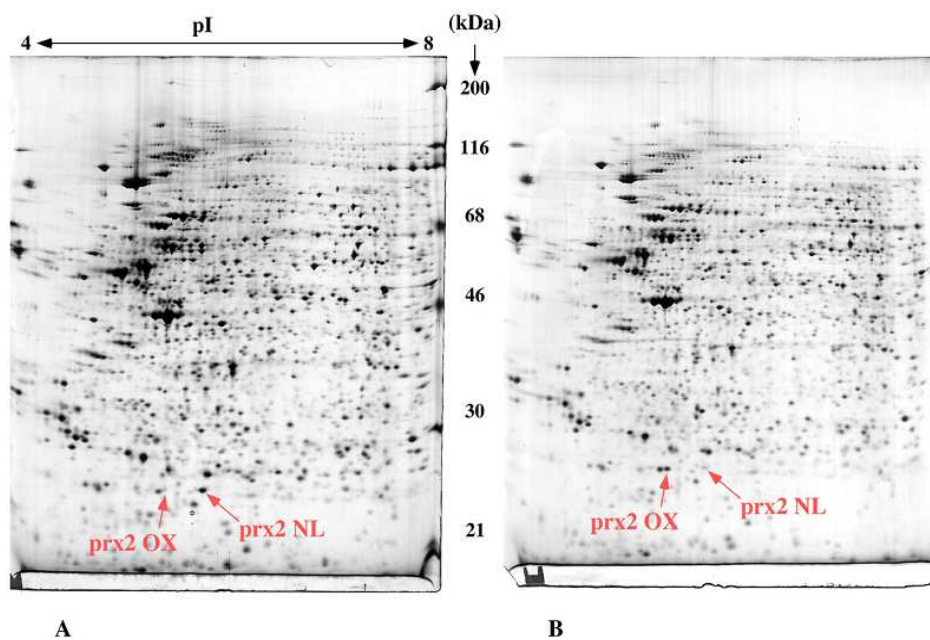


Fig. 1. Detection of proteins as well as modification of which by combining 2DE protein separation and mass spectrometry based identification. The figure represents peroxiredoxin and its modified form in Jurkat cells in normal cell culture conditions (panel A) or stressed with t-Butylhydroperoxide. Cells were lysed, and extracts were separated by two-dimensional electrophoresis: Linear pH gradient 4 – 8 was used for IEF first dimension and SDS-PAGE was used for second dimension. Gels were subsequently stained with silver-nitrate. Arrows indicate the position of the normal (prx2NL) and the oxidized (prx2OX) forms of peroxiredoxin 2. The change in pI (0.25 pH units) is due to the sole oxidation of the -SH group in peroxiredoxin 2 (adapted from Rabilloud, et al [4]).

3. Low abundance proteins and organelle proteomics

Although large-scale proteome analysis provides valuable information regarding cultured cells, tissues, or body fluids; nevertheless, analysis of the whole proteome might be too complex even at cellular level [5]. There are many proteins with low copy number, which might be far from resolution of the current methods when the whole tissue or cell is used as the source of proteins. Such proteins require enrichment before analysis. Considering the large variation in the expression level of proteins within a cell or tissue, the low abundance proteins become inevitably masked by high abundance proteins [6]. It should be noted that most regulatory proteins such as kinases, GTPases, certain membrane receptors, polymerases as well as transcription factors are present in low copy number. As a result an important layer of information would be lost [7]. This becomes even more important when

there is only limited amount of material available for analysis (e.g. medical biopsies). Although genomic approaches have benefited from amplification methods such as polymerase chain reaction, protein based methods are poor in this regard as there is not currently any method available for their amplification. This drawback was solved in part by availability and application of accurate methods of identification such as mass spectrometry that require infinitesimal amount of proteins and to some extent using enrichment methods of low abundance proteins of interest by methods such as chromatography. For example, the total protein content of cells can be enriched by fractionation methods based on affinity procedures to isolate groups of proteins displaying similar features (lectin based isolation of glycoproteins, charge and hydrophobicity based protein separation or application of specific antibodies for isolation of phosphoproteins, etc.). This procedure has the advantage of simplifying the complexity of crude cell or tissue extracts, thereby maximizing the probability of detecting low abundance proteins. However, it should be noted that information with regard to the location of the protein of interest in the cell, organ or tissue remains to be elucidated. Organizing eukaryotic cells into sub-compartments with specialized features and functions; the organelles, provides a unique opportunity to link proteomics data with functional units. In addition identification of a specific protein within a specific organelle not only would be a step forward in our understanding of the function of the protein of interest and the molecular mechanisms in which it is involved but also the functional features of the related organelle as well. So far protein profile of several organelles have been elucidated by proteomic [5,7].

4. Proteomics in cancer research

Cancer is well known for its complex nature, which results from accumulation of numerous molecular alterations altogether lead to genetic instability, cellular proliferation, and acquisition of invasive phenotype and metastasis. While it has long been known that cancer to be a genetic disorder, but at functional level, it is rather a protein and proteomics related disease, since tumor progression, invasion and metastasis all depend on functional identity of cells or proteins such as growth factors, transcription factors, enzymes, signal transducers, proteases, etc. As a result different kinds of drugs that target different cellular components either protein [8] or nucleic acid constituent of the cells have so far been designed for treating the disease. Nonetheless such a broad range of drug could not lead to a satisfactory progress in complete treatment of the disease.

Despite our deeper understanding of the alterations and aberrations that happens in cancer cells along with advances in characterizing diversity of cancer transcriptomics, proteomics has the potential to complement further expansion the wealth of present information generated by genomics in cancers from different aspects. These aspects include; (i) there is generally not a direct correlation between level of transcription of specific genes and relative abundances of their corresponding proteins, since the resultant transcript might be subject of degradation, inactivation, or being kept in silent and inactive form until the time it is required (RNA granules) [9] (ii) due to the differential splicing and translation, each gene may encode several different protein variants with different properties; (iii) the key proteins driving malignant behavior of cancer cells can undergo post-translational modifications including phosphorylation, acylation, and glycosylation; and (iv) proteome reflects dynamic

changes, it could be a suitable indicator of the disease progression and could be used for monitoring and following up the course and response of the disease to the therapy. And finally proteins represent the more accessible and relevant therapeutic targets [10,11].

Despite efforts and successes in prevention of cancers through applying screening programs along with public awareness, changes in habits, and application of better treatment strategies as well as postoperative programs, nevertheless, there wasn't so far prospect for a long life in case of many patients and cancer still remains the major cause of patients' death. The major cause of cancer death is metastasis. In most cases diagnosis of cancer and treatment of which is done when tumor has been developed well, metastasis has happened, and tumor has spread into distant organs. Proteomics could play important role not only in the study of molecular mechanism of carcinogenesis but also discovery of new cancer markers for early diagnosis of the disease, staging in addition to evaluating prognosis, prediction and monitoring of patients' response to a particular therapy. Such markers could directly be released from cancer cells or may represent as part of host's response to malignancy. They might be released into body fluids, which make their detection easy. Cells most often shed proteins into extracellular fluids including interstitial fluids, lymph and blood plasma. Whilst tissue interstitial fluids are in direct contact with tissue/cells via transfer of molecules, the composition of blood plasma results from its interaction with tissue's interstitial fluids. Blood plasma is dynamic; it influences the composition of other body fluids and becomes influenced by body fluids as well. It is important to realize that relative concentration of biomarkers is highest in the tissue of origin and surrounding interstitial fluid. However during the course of drainage from interstitial tissues' fluid into the lymph and lymph vessels and then into blood vessels the concentration of biomarker may become subject of crucial reduction. As a result, the concentration of specific biomarker in blood would significantly be lower than its original concentration in the interstitial fluid. Nevertheless, various body fluids represent more or less rich source of different types of biomarkers [10,11].

5. Proteomics of esophageal carcinoma

With 386000 annual death, esophageal cancer is the sixth leading cause of cancer death worldwide [12] [13]. The incidence of esophageal cancer is geographically diverse as a large variation could be observed for different parts of the world. The high incidence of esophageal cancer in certain parts of the world indicates a role for environmental as well as habitual factors in addition to genetics.

While reports indicate the highest incidence rate of esophageal cancer for northern Iran and certain parts in China, however, there are other high incidence areas in the world most of which are located in the Asian esophageal cancer belt. The Asian esophageal cancer belt consists of the central and eastern Asian countries including; Turkmenistan, Uzbekistan, Karakalpakstan (an autonomous republic in the eastern part of Uzbekistan), Kazakhstan, and parts of Turkey. Together these high-risk geographic areas appear to extend from north-western Iran to China, along the path of ancient Silk Road collectively known as "Central Asian Esophageal Cancer Belt" (Figure 2) [14].

Despite recent increase in the rate of esophageal adenocarcinoma in Western world, esophageal squamous cell carcinoma (ESCC) still remains the most prevalent subtype of esophageal cancer [16]. With five-year survival rate as less than 10% prognosis of

esophageal cancer still remains poor. A primary cause for such high mortality is the fact that in most cases ESCC could be detected very late when tumor has developed well, invaded

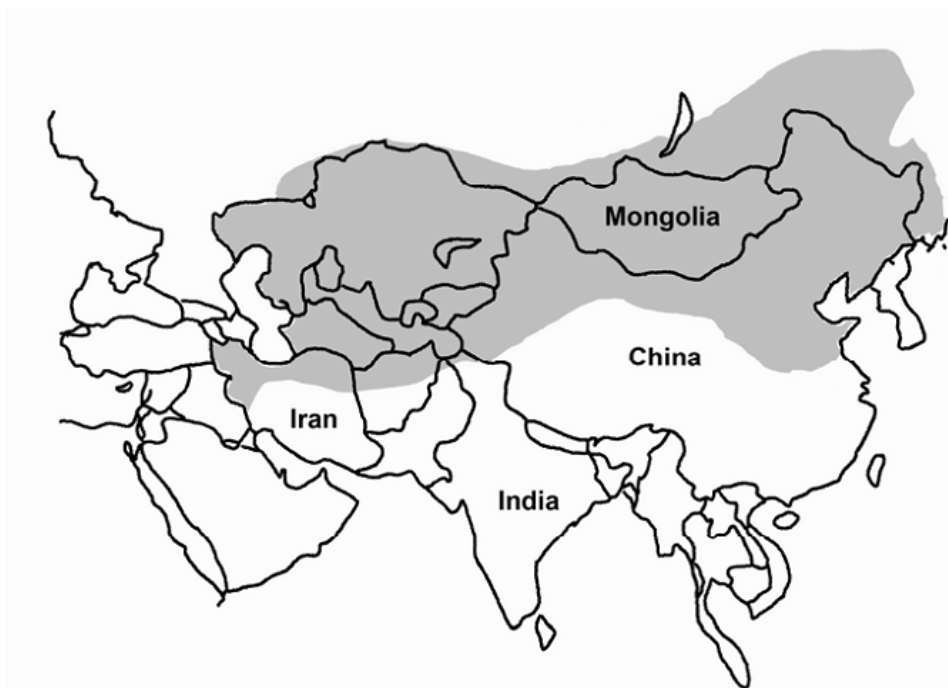


Fig. 2. The Asian esophageal cancer belt. The belt starts from eastern Anatolia in Turkey [15], extends through Iran to central Asian countries, China [14] and to the far east.

surrounding tissues and organs, and therefore at an advanced stage of the disease. Surgical resection has shown to be ineffective in 40%-60% of cases due to low resectability of the disease, the presence of distant metastases, in addition to high operation risk. Additionally, the conventional chemo and radiotherapies are relatively ineffective which further account for the poor long-term survival. The patient's survival becomes poor when the tumor spreads and extends through esophageal wall or when it is diagnosed with the widespread involvement of lymph nodes. Thus, early diagnosis and exact histological grading of the ESCC are critical for therapeutic management [17]. Over the past years, the molecular etiology of esophageal cancer was subject of extensive researches. Multiple genetic alterations, such as loss of tumor suppressor genes and activation of oncogenes were found to be associated with the development of esophageal cancer [18,19].

Although recent advancements such as microarray in addition to traditional molecular methods have been used for screening ESCC in order to find the important molecular alterations that ultimately result in ESCC [20-22], nevertheless, thus far target biomarkers applicable for the detection and therapeutic strategies and genes to act as molecular targets have not been well identified, indicating further limitations in the effective treatment of

ESCC. The high throughput and sensitive proteomic technology is hoped to open an effective venue for screening the novel cancer specific biomarkers for ESCC. Tissue and cell line based proteomics have widely been used in the study of ESCC and so far protein markers (biomarkers) have been identified as potential biomarkers for diagnosis of ESCC and possible follow-up of the treatment. Most of such identified protein molecular markers are those that are involved in cytoskeleton organization, metabolism, differentiation, apoptosis, cell growth, and metastasis as well as redox reactions. In subsequent sections, we present a summary of the recent achievements applying proteomics.

6. Cytoskeleton

Actin network is essential for several important cellular functions such as pseudopodia formation, motility, division, cell surface receptor movement, anchorage, and contact inhibition. During malignant transformation, alteration in the expression of actin microfilament network as well as other actin-associated proteins which are involved in the morphological changes and cytoskeletal organization could be seen. Among such proteins are tropomyosins (TPMs). As a major structural component of cytoskeletal microfilaments, multiple isoforms of TPMs were identified in the cultured non-muscle cells. At expression level different isoforms of TPM are regulated dissimilarly in tumors, implying that these isoforms may have different functions in cell transformation. TPM1 [23] and TPM2 [24] have shown to be subject of down-regulation while TPM4 [23] and TPM3 ([25], and as our unpublished result indicates (in a study on cell lines)) are significantly subject of up-regulation in ESCC tissues. In addition, fusion of TPM4-ALK was observed to happen in ESCC [24] which results in the up regulation of anaplastic leukemia kinase. Though as a cytoskeletal and housekeeping protein TPM4 promoter is constantly active, the fusion protein (TPM4-ALK) is constantly expressed in tumor cells which results in up regulation of fused anaplastic leukemia kinase in the cell and its oncogenic outcome. Deregulation of TPM isoforms may cause an imbalance in the normal phenotype of epithelial microfilaments, which leads to malignant phenotype of the aberrant cells. These alterations may provide clues for the early detection, diagnosis, and identification of therapeutic targets. In addition to TPMs altered expression of members of myosin family of proteins have also been reported in ESCC ([24] and our unpublished results).

Transgelin, a calponin related protein whose expression was observed to change in transformed cells is another member of cytoskeletal associated proteins that remarkably increases in ESCC. Distinct types of transgelin isoforms presents exclusively in cancer tissues [23]. Transgelin is an actin microfilament binding protein whose expression is regulated by deregulated Ras expression in a Raf independent pathway of transformation. Loss of transgelin in breast and colon tumors and in RIE-1 cells has also been reported [26].

Keratins are components of intermediate filaments of cytoskeleton functioning especially in epithelial cells. Keratin1 and keratin 8 ([24], as well as our unpublished results on ESCC cell lines, [27-29]) and keratin 13 were observed to be overexpressed in ESCC, while keratin 4 and keratin 14 are down regulated [30] in.

Desmin is another member of intermediate filaments that subjects to down regulation in ESCC [16]. As a 52 kDa protein, desmin is a subunit of intermediate filaments in the tissues

of skeletal, smooth, and cardiac muscle [31] cells. While it is a muscle cell marker and important in muscle cell's development, nevertheless its exact role is not yet known for other cell types and demands further studies to unravel its true function.

Another actin binding protein is fascin that overexpression of which was observed in ESCC. Since overexpression of fascin was found to be associated with significant increase in the motility and dynamics of cell lines [16]; it could be concluded that the same consequences which are; the increased invasion and metastatic potential to happen for ESCC too.

α -actinins are actin binding and cross-linking proteins. Expression of alpha actinin 4 (ACTN4) was shown to increase progressively from stage I to stage III. Clinico-pathological correlation using TMA (tissue microarray) revealed that overexpression of ACTN4 is significantly associated with the advanced tumor stage and lymph node metastasis [25] in ESCC.

In addition gamma actin, tubulin alpha-1 chain, and tubulin beta-5 chain were also reported to be subject of change in expression in ESCC. Overexpression of these proteins was reported in ESCC [23].

7. Differentiation

Data obtained from models of carcinogenesis suggest that alteration in the normal differentiation process is associated with neoplastic transformation [16]. As a result, altered expression of proteins, which are related to differentiation, is expected to play role in carcinogenesis through dedifferentiation, resistance to terminal differentiation, or alteration of differentiation.

S100A8 ([32], and our unpublished data) and S100A9 [16] are the two other calcium binding proteins which are associated with the myeloid cell differentiation. These two proteins are subject of down regulation in ESCC. Recently, S100 family of proteins have received increasing attention as their possible involvement in several human diseases, including cancer.

Annexins [33,34] were shown to play important role in esophageal carcinogenesis. Annexin I down regulation has reportedly been observed in ESCC ([16, 35, 36], as well as our unpublished results). Loss of annexin AI correlates with the early onset of tumorigenesis in esophageal carcinoma. It was found that expression of annexin AI to be correlated with the differentiation status of esophageal carcinomas as high expression of annexin AI was reported to occur in the poor differentiated ESCC [35,36]. In addition to annexin AI, down-regulation of annexin A II and overexpression of annexin AIX were also observed in our studies on ESCC (unpublished results) and others [37-39].

Transglutaminases (TGases) are calcium dependent enzymes that catalyze formation of isopeptide bonds between amide group of glutamine and the ϵ -amino group of lysine during the process of terminal differentiation in stratified squamous epithelia. It was shown TGases to be subject of down regulation in ESCC [16,25]. Among TGases, the protein-glutamine gamma-glutamyltransferase E (TGM3) plays key role in epidermal terminal differentiation through cross-linking structural proteins such as involucrin, loricrin, and small proline-rich proteins. Although the role of TGM3 in the differentiation of skin

keratinocytes has been well established, however, little information is available regarding its involvement in esophageal epithelium. TGM3 stabilizes the cornified envelope of the cells, a process that precedes the transition of keratinocytes to corneocytes by apoptosis [40, 41, 42]. It is among important molecules involved in the adhesion which is expressed by epithelial cells and regarded as inhibitor of invasion. As a result downregulation or loss of TGM3 correlates with dedifferentiation, increased invasion and high incidence of lymph node metastasis [43]. Although the role of TGM3 has well been established in the differentiation of skin keratinocytes, nonetheless, little information is available regarding its involvement in esophageal epithelium transformation. In addition to esophageal carcinoma, downregulation of TGM3 was also reported in laryngeal carcinoma [44], as well as head and neck squamous cell carcinoma [45].

The other protein that reports have indicated its low expression in ESCC is galectin7, a member of the galectin family. The low expression of this protein in ESCC is consistent with a differentiation defect in keratinocytes [16]. The major functions of galectin7 include regulation of cell to cell and cell to matrix interactions, apoptosis and immunity. It should be noted that both downregulation (above) as well as upregulation of galectin7 was reported with regard to ESCC. Upregulation of galectin7 was reported by Zhu et al (2010) in ESCC tissues [17].

Epidermal-type fatty acid-binding protein (E-FABP) is a member of the FABP family that mediates transport and utilization of fatty acids. FABPs are small cytosolic non-enzymic proteins that have tissue specific expression. They are involved in fatty acid signaling, cellular growth and differentiation. It was proposed that they play a role in cellular lipid uptake and transport, metabolic pathway, and regulation of protein metabolism. Downregulation of E-FABP in has been reported for ESCC [46].

8. Metabolism

Several proteins that are involved in cellular metabolism undergo overexpression in ESCC, for example, AKR (aldo-keto reductase) family 1, reflecting an increased metabolic and biosynthetic requirement of tumor cells and their possible involvement in carcinogens metabolism. AKR members have shown to be involved in carcinogen metabolism. As an example AKR can activate polycyclic aromatic hydrocarbons (PAHs) by oxidizing trans-dihydrodiol proximate carcinogens to reactive and redox active ortho-quinones. PAHs are ubiquitous environmental pollutants and human carcinogens. Overexpression of AKR might yield more active carcinogens and result in cellular transformation and tumor development in ESCC [16,47].

Glutathione transferases (GSTs) compose multigene family of dimeric enzymes of phase II detoxification that catalyze conjugation of glutathione to the lipophilic substrates in order to make them more water soluble or electrophile [48] essential for their excretion from the body. Since a large proportion of pro-mutagens and pro-carcinogens are lipophilic compounds, by conjugating them with the electrophilic glutathione they become more water soluble and easier targets for excretion into bile or urine. GSTs can be induced by many of their substrates and by some non-substrate compounds as well. For example, butyrate, an important luminal component produced from bacterial fermentation of dietary fibers, is an efficient inducer of GSTs in colonic carcinoma cell lines. M and P family of GSTs have a regulatory role in mitogen-activated protein (MAP) pathway and resistance to drugs.

In addition, overexpression of GSTs is associated with the increased resistance to apoptosis that could be initiated by various stimuli [49]. GSTM1, GSTP1 and GSTT2 are expressed in esophagus mucosa. Higher expression of GSTP1 has been observed in esophagus compared to other GSTs [50]. GSTM2 was found to be over-expressed in ESCC. The overexpression of this enzyme in ESCC might be a response to the increased GSTM substrates or to the bacterial metabolites in the esophagus. In addition to GSTM2, overexpression of GSTP was also reported in ESCC [16,51].

Alpha enolase is a multi-functional enzyme in the glycolytic pathway which catalyzes formation of phosphoenol pyruvate from 2-phosphoglycerate. The expression of alpha enolase was seen to be elevated in ESCC tissues [52] that might indicate a higher metabolic rate as well as switch to glycolytic pathway as possibly the main source of providing the required energy.

Another metabolism related protein whose expression is affected by cancer is glutamate dehydrogenase (mitochondrial) GLUD1. The enzyme is involved in glutaminolysis that is important in cancer metabolism [53]. Our observation in the cell lines prepared from ESCC also indicates that GLUD1 subjects to down regulation in ESCC.

9. Redox reaction

Accumulating evidences indicate that intracellular redox state plays important roles in cellular signal transduction and gene expression [54]. Reactive oxygen species (ROSs) which are produced during physiological processes in response to external stimuli, can affect intracellular redox state. At low levels, ROS modulate gene expression through modulating cellular redox state, however, at higher levels ROSs are extremely deleterious and potentially damage DNA, proteins, carbohydrates, and lipids. It has been suggested that ROSs play roles in all stages of carcinogenesis, including initiation, promotion, and progression [55,56]. In order to protect cells from oxidative radical stress, cells have developed defense systems that comprise proteins superoxide dismutases (SODs), catalase, glutathione peroxidases, and peroxiredoxins (PRXs). The up-regulation of MnSOD and PRX1 in ESCC and their linear correlation with progression of disease from premalignant to invasive cancer reflect the cell defense effort in maintaining intracellular homeostasis. Interestingly, a minor down-regulation of PRX2 isoform was detected in ESCC [23,24] suggesting that different PRX isoforms may have slightly different functions unique to the esophageal neoplasms [23]. We observed PRDX5 overexpression in ESCC (unpublished observation). Thioredoxin peroxidase (TxP) uses thiol groups as reducing equivalent donors to scavenge oxidants. By reducing reactive oxygen species formation, TxP inhibits caspase activity and hence apoptosis. Overexpression of TxP in ESCC may increase the number of proliferating ESCC cells by inhibiting apoptosis [16].

10. Heat shock proteins

Heat shock proteins are the highly conserved cytoprotective proteins in all species. They play essential role in protein folding, transport, translocation, degradation, and assembly, even under unstressed conditions. GRP78 is an endoplasmic reticulum (ER) chaperone calcium binding protein. It is involved in many cellular processes including the translocation of newly synthesized polypeptides across the ER membrane, facilitation of the folding and assembly of newly synthesized proteins, degradation of misfolded proteins

through proteasome, and regulation of calcium homeostasis. In addition to above functions GRP78 endows cancer cells ability to resist against anticancer drugs such as chemotherapy, antiangiogenesis antibodies, and anti hormonal therapy. It was shown to be involved in tumor cell immune resistance, proliferation and metastasis added to its role against apoptosis. Thus, it is reasonable that its overexpression accompany with the increased rate of carcinogenesis. In accordance with these properties, elevated expression of GRP78 could be observed in ESCC. ESCC patients with higher expression of GRP78 show a shorter survival than those with low or no expression of GRP78 [52,57].

Calreticulin is another calcium binding endoplasmic reticulum specific protein whose up regulation was observed in ESCC [24,52]. It is involved in the regulation of intracellular calcium homeostasis and endoplasmic reticulum calcium storage capacity [52,58]. Calreticulin is a lectin that interacts with the nascent and newly synthesized glycoproteins. It functions as a molecular chaperon during folding of glycoprotein [59]. It cooperates with calnexin, glycoprotein glucosyltransferase and glucosidase in calnexin/calreticulin cycle of protein folding. Role of this cycle is engagement in selective folding of newly synthesized glycoproteins in the process of protein translation [60,61]. Approximately all glycoproteins transiently interact with one or both of these two proteins (i.e. calnexin or calreticulin) during maturation or degradation after misfolding [62,63].

AlphaB-Cryst is a member of the small heat shock proteins (HSPs), which are ubiquitous chaperone molecules related to stresses. They bind to partially denatured proteins, dissociating protein aggregates, modulating the correct folding, and cooperating in transporting newly synthesized polypeptides to the target organelles. AlphaB-Cryst is able to inhibit both mitochondrial and the death receptor apoptotic pathways through abolishing the autoproteolytic maturation of the partially processed caspase-3 intermediate. Intriguingly, while other HSPs were usually up-regulated in tumors, alphaB-Cryst was often down-regulated in various cancers including in ESCC tissues. These results point out that alphaB-Cryst plays a role distinct from other HSPs in the carcinogenesis and its underexpression might candidate it as a general tumor marker for various types of cancers [23].

gp96 and Hsp27 are the two other chaperones whose expression change have been reported in esophageal cancer. Reports indicate upregulation of gp96 and down regulation of Hsp27 in ESCC. Hsp27 and gp96 are stress-response proteins. gp96 also plays a role in tumor immunity [16]. In addition to gp96, overexpression of HSP70 has also been reported in ESCC [24].

11. Cell growth

Several cell growth related proteins' expression was seen to change in ESCC. PCNA is among such proteins whose overexpression could be observed in ESCC [16]. As a highly conserved protein in eukaryotes, it is essential factor for DNA replication and DNA repair. In addition to PCNA, upregulation of DNA directed RNA polymerase B has formerly been reported by our group in ESCC [24].

RNA binding motif proteins 8A (RBM8A), the other growth related protein is also overexpressed in ESCC [16]. RBMs play key role in post-transcriptional regulation of gene expression in eukaryotic cells and mediate mRNA processing including terminal processing of which; intron splicing, editing and deamination of nucleotides [64].

Clusterin, the so-called testosterone repressed prostate message, sulfated glycoprotein, complement associated protein SP-40, and complement cytolysis inhibitor, is an 80-kDa heterodimeric highly conserved secreted glycoprotein expressed in a wide variety of tissues and was found in all human fluids. It responds to a number of diverse stimuli, including hormone ablation and has been attributed to function in several diverse physiological processes such as sperm maturation, lipid transportation, complement inhibition, tissue remodeling, membrane recycling, cell adhesion and cell- substratum interactions, stabilization of stressed proteins in a folding competent state and is involved in promotion or inhibition of apoptosis. In addition, loss and downregulation of clusterin in ESCC, it was also lost or decreased in tumor cell lines and tissues [65].

Another potential tumor suppressor protein is prohibitin that was found to be differentially expressed in cancerous tissues compared to the adjacent normal epithelium. Interestingly, while expression of prohibitin is positively correlated with the progression of precancerous lesions, however, it is inversely correlated with the differentiation grade of squamous cell carcinoma of esophagus. The expression of prohibitin drops with dedifferentiation of ESCC. This pattern of expression implies that prohibitin may play different roles in different stages of esophageal tumorigenesis [23].

Eukaryotic translation initiation factor 1 a (eIF-1a), reticulocalbin and transmembrane protein 4 are three other proteins that overexpress in ESCC [16]. eIF-1a stabilizes Met-tRNA to the 40S ribosomal subunit, thus prevents pre-maturation association of 40S ribosomal subunit to 60S subunit of ribosome [66]. eIF-1a along with other eIFs stimulate decoding of AUG start codon in mRNA [67].

Reticulocalbin is a calcium binding protein located in the endoplasmic reticulum lumen. Overexpression of this protein plays a role in tumorigenesis, tumor invasion and resistance to drug [68,69].

12. Metastasis

Cancer cells escape the primary tumor mass and penetrate into the surrounding tissues or tissues at far distant through the process of invasion and metastasis, the two processes that require degradation of extracellular matrix and/or basement membranes. The key molecules involved in the degradation of these structures are cysteine, serine, and aspartic acid protease as well as matrix metalloproteinases (MMPs). MMPs contains collagenases (MMP-1, MMP-8, MMP-13, MMP-18), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11) matrilysins (MMP-7, MMP-26) mating type (MMP-14, MMP-15, MMP-16, MMP-24, MMP-17, MMP-25) and non-classified (RASI-1, enamelysin) [70]. Generally, gelatinases are more often observed in tumor tissues. It seems that they are involved more in the invasion rather than other members of matrix metalloproteinases [71,72]. High expression of MMP-1 [73], MMP-7 [74], MMP-11 [75] is associated with the worse prognosis of tumors, while MMP-9 and MT1-MMP are involved in the depth of invasion [76,77]. MMP-2 and MMP-3 were found to be correlated with the lymph node metastasis in ESCC [78].

Down-regulation of neutrophil elastase inhibitor and SCCA1 (below) in ESCC was among other observation by proteomic studies. Neutrophil elastase is an inflammatory protein that is

mainly produced by neutrophils. The protease degrades extracellular matrix thereby increases ability of neutrophils to infiltrate into the tissues. Neutrophil elastase might also be released by some cancer cells to serve a similar function. Low expression of elastase inhibitor in ESCC would result in an increased enzyme activity, facilitating tumor invasion and metastasis.

Squamous Cell Carcinoma Antigens (SCCAs), are members of serine protease inhibitors (serpins) [79] superfamily that strong expression of which could be observed in different epithelial cancers. Two different isoforms of SCCA are encoded by two highly homologous genes SCCA1 and SCCA2 [80]. Both the SCCA1 and SCCA2 proteins are physiologically present in the suprabasal layers of normal stratified squamous epithelium[81]. SCCA1 [23] was shown to inhibit papain like cysteine proteinases, cathepsin S, K, and L. Serpins are involved in the multiple cellular biological processes including tumor cell invasion, cellular differentiation, and apoptosis. SCCA1 may function intra as well as extracellularly, serving as a cytoprotective mediator [16,23].

APA-1, a zinc finger protein, was shown to be overexpressed in ESCC. APA-1 is a transcription factor which activates transcription of matrix remodeling genes such as matrix metalloproteinase 1 (MMP-1) during fibroblast senescence [52]. The same role for APA-1 overexpression could be envisaged for esophageal cancer.

13. Apoptosis

Apoptosis is a major barrier for cancer cells that they must have to overcome in order to survive. The modest increase observed in COX-2 and p53 protein expression with progression from normal to dysplasia suggests that these markers may be the most informative in the more advanced state of neoplasias [17,30]. Cyclooxygenases (Cox-1 and Cox-2) are enzymes which are involved in the formation of prostaglandins from arachidonic acid. While Cox-1 is constitutively expressed; Cox-2 is induced by cytokines, tumor promoters, growth factors and viral induced transformation. Cox-2 was found to be expressed in various malignant tumors [82]. Expression of Cox-2 could be induced by p53. In turn, Cox-2 negatively affects p53 activity through physical interaction with p53. Cox-2 is a positive regulator of growth while p53 is a negative regulator of growth thus increasing expression of Cox-2 by p53 seems to be a controlling event of growth by creating a balance between induction and inhibition of cellular division. It is suggested that p53-dependent induction of Cox-2 abate apoptotic and growth inhibitory effect of p53 [83].

14-3-3 protein sigma, also known as stratifin or HME-1, has recently reported to be down regulated in ESCC. It is transactivated by p53 in response to DNA damage and negatively regulates both G1/S and G2/M cell cycle progression. Overexpression of stratifin increases stabilization of p53 through blocking Mdm2 mediated p53 ubiquitination and enhanced oligomerization of p53, leading to the increased p53 transcriptional activity. Additionally, expression of stratifin inversely correlates with the differentiation grade of ESCC indicating that malignant cells arising from esophageal epithelium may lose stratifin in progressive dedifferentiation [23]. Stratifin is a checkpoint protein that causes G2 arrest following to DNA damage. Inactivation of this protein; mainly by methylation, was reported in some tumors. Likely loss of this protein impairs the function of G2/M checkpoint results in the accumulation of genetic defects and ultimately cancer [84].

Table 1 represents proteins that were identified using proteomic based methods in ESCC and we discussed here. These are not the only proteins identified with regard to ESCC, though other proteins could also be found in other literatures. Here we focused on some proteins as examples for documenting the applicability of proteomic based methodologies in the molecular etiology of cancers and among which esophageal cancer in particular. A long list of proteins for ESCC could be found in literatures that are far from scope of the present book chapter. We propose that in future studies attempts to be focused on narrowing down the list of proteins to as small number and to as tissue specific as possible till each of such proteins could be correlated with a specific type of cancer. Such a narrowing down is important from that respect that makes detection and prediction of specific type of cancer possible before the onset of the disease, especially by using such proteins as markers in body fluids, body secretions and excretions and other rout of discharge from the body. Fortunately, recent reports indicate the potential of proteomic based studies in correlating and establishing fine relationship between the expression patterns of several proteins with the stage of carcinogenesis as well as differentiation or grade of cancer. Among such reports are papers published by Qi and colleagues [23] and Nishimori, et al. [85] that showed well such a correlation. These proteins have significant clinical value since they could be used as molecular markers in order to evaluate the tumor per se and prognosis for evaluating the efficacy of the treatment as well as prediction of recurrence, etc. (figure 3).

Classification (cellular function)	Upregulated	Downregulated
Cytoskeleton	TPM4 TPM3 Transgelin Keratin 1 Keratin 8 Keratin 13 Tubulin alpha chain 1 Fascin Tubulin Beta-5 chain Alpha actinin 4	TPM1 TPM2 Keratin 4 Keratin 14 Desmin
Differentiation	Annexin A4 Galectin 7	S100A9 S100A8 Annexin A1 Annexin A2 TGM3 Galectin 7
Metabolism	AKR GSTM2 Alpha enolase	GLUD1
Redox reaction	MnsOD PRX1 PRX5 TxP	PRX2

Classification (cellular function)	Upregulated	Downregulated
Heat Shock Protein	GRP78 Calreticulin Gp96 Hsp70	$\alpha\beta$ -Cryst Hsp27
Cell growth	PCNA DNA direct RNA Polymerase B RBM8A Prohibitin eIF-1A Reticulocalbin Transmembrane protein 4	Clusterin
Metastasis	MMP-1 MMP-7 MMP-11 MMP-9 MT1-MMP MMP-2 MMP-3 APA-1	SCCA1 Neutrophil elastase inhibitor
Apoptosis	Cox-2 P53	14-3-3 Protein Sigma

Table 1. Proteins identified by proteomics in ESCC and discussed in the text.

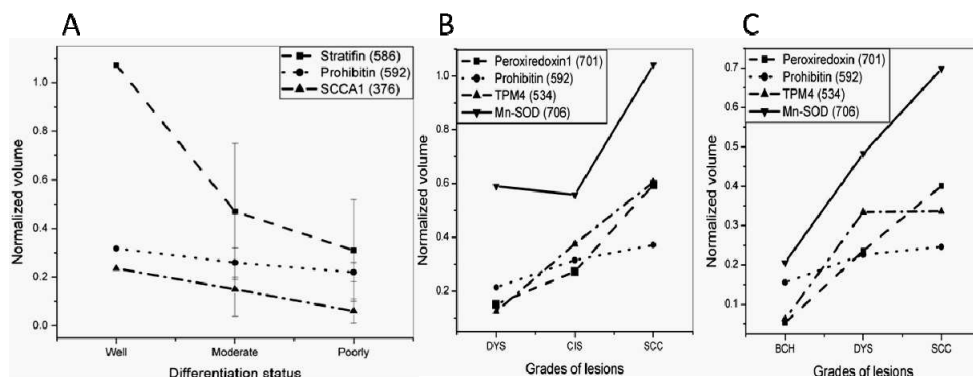


Fig. 3. Correlation between expression patterns of several proteins identified by 2DE and differentiation status (A), grade (B) or the degree of lesion (C) in ESCC. Numbers in the parenthesis indicate the spot number in 2DE gel pattern explained in the text by the authors. A well correlation could be established for stratifin, TPM4, peroxiredoxin and the other member of redox family of proteins; peroxiredoxin1, as well as Mn-SOD with the degree of

the disease progression. Other proteins show more or less the same pattern of expression change (adapted from Takanori Nishimori, et al [85]).

14. Conclusion

The complexity of the carcinogenesis in addition to the dynamics of the protein constituent of the cells demand new approaches for the analysis of the molecular etiology of cancers and among which ESCC for establishing appropriate strategies of their successful detection, treatment, and follow up. Proteomics is a powerful mean of gene expression analysis applicable both at translational as well as posttranslational level. In addition it could be used for studying protein-nucleic acid, or protein-drug interaction, along with vast other applications. As a result, proteomics could be an appropriate complement for the gene expression based analyses. It enables to put further steps of information ahead which is the entire genes being expressed in a cell or tissue at a given time and under specific condition. So far many proteins in different steps of carcinogenesis have been identified that found to be subject of alteration in carcinogenesis. Accumulating data indicate that proteomics could be an efficient approach for the identification of molecular alterations in ESCC carcinogenesis in addition to other cancers.

The data generated by proteomics in ESCC has so far lead us to the identification of a set of proteins, which are involved in the different stages of ESCC carcinogenesis. These proteins are not only related to the alterations in the structure but also to the function of ESCC among which cell growth and division along with apoptosis and invasion. Moreover, several of such identified proteins were also found to be as appropriate biomarkers of ESCC, which authenticates the efficacy of the proteomic based strategies in clinical investigations and practical application. It is expected that proteomic evaluation of tissues and body fluids could open a venue to the achievement of the proper approaches of the assessment of the overall status of health and prognosis of cancers including ESCC.

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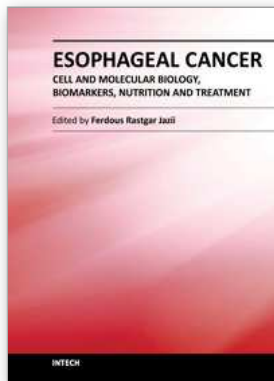
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Esophageal Cancer illustrates recent achievements and investigations in the esophageal tumorigenesis from different perspectives. Readers find mechanisms involved in esophageal tumorigenesis, cellular, molecular, genetic, epigenetics, and proteomics, their relevance as the novel biomarkers and application in esophageal cancer diagnosis and therapy. The book covers detailed effect of nutritional factors in addition to ethanol metabolic pathway in the inhibition of retinoic acid metabolism and supply. Diagnosis, classification, and treatment of esophageal cancer, application of both surgical and non surgical methods as well as follow up of the disease are described in detail. Moreover readers are endowed with especial features of esophageal cancer such as multiple early stage malignant melanoma and pulmonary edema induced by esophagectomy, the two features that received less attention elsewhere in literature.

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