

Quantitative tissue proteomics of esophageal squamous cell carcinoma for novel biomarker discovery

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Abbreviations: ESCC, esophageal squamous cell carcinoma; *PSAP*, prosaposin; *PDIA*, protein disulfide isomerase; *PLEC1*, plectin 1; *POSTN*, periostin; iTRAQ, isobaric tags for relative and absolute quantitation

Esophageal squamous cell carcinoma (ESCC) is among the top ten most frequent malignancies worldwide. In this study, our objective was to identify potential biomarkers for ESCC through a quantitative proteomic approach using the isobaric tags for relative and absolute quantitation (iTRAQ) approach. We compared the protein expression profiles of ESCC tumor tissues with the corresponding adjacent normal tissue from ten patients. LC-MS/MS analysis of strong cation exchange chromatography fractions was performed on an Accurate Mass QTOF mass spectrometer, which led to the identification of 687 proteins. In all, 257 proteins were identified as differentially expressed in ESCC as compared with normal. We found several previously known protein biomarkers to be upregulated in ESCC including thrombospondin 1 (*THBS1*), periostin 1 (*POSTN*) and heat shock 70 kDa protein 9 (*HSPA9*) confirming the validity of our approach. In addition, several novel proteins that had not been reported previously were identified in our screen. These novel biomarker candidates included prosaposin (*PSAP*), plectin 1 (*PLEC1*) and protein disulfide isomerase A 4 (*PDIA4*) that were further validated to be overexpressed by immunohistochemical labeling using tissue microarrays. The success of our study shows that this mass spectrometric strategy can be applied to cancers in general to develop a panel of candidate biomarkers, which can then be validated by other techniques.

Introduction

Gastrointestinal malignancies are among the frequently occurring tumors in humans. Esophageal cancer has been reported to be the eighth most common malignancy worldwide.¹ It can be classified into two histological types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. The incidence rate of ESCC is higher as compared with esophageal adenocarcinoma in developing countries being especially common in certain areas of China, Iran and India.² The incidence and mortality rates are 2- to 3-fold higher in males than females.³

The carcinogenesis of ESCC is a multifactorial and multistep process. It involves various genetic and environmental factors. A number of risk factors have been associated with the development of ESCC. Alcohol and tobacco are known to be major risk factors. Others include diet deficient in vitamins,⁴ polyaromatic hydrocarbons in smoked foods and consumption of extremely hot beverages.⁵ Other predisposing factors for ESCC include Plummer-Vinson syndrome, tylosis⁶ and lye-ingestion. Surgical resection of tumors is the standard mode of treatment in early stages of ESCC. However, ESCC patients are often diagnosed when the cancer is at an advanced stage when surgical intervention

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is not an option and chemotherapy as well as radiotherapy are the mainstay in the treatment of the majority of cases.

Dysphagia is the most common symptom in patients with ESCC and appears late in the course of the disease. The lack of early clinical symptoms and poor sensitivity of the molecular markers is a major reason for late diagnosis resulting in poor survival. Over the past several years, attempts have been made to identify molecular markers for the diagnosis of ESCC. In a proteomic study, Zhu et al. showed that galectin 7 is upregulated in ESCC tissues.⁷ Other proteomic studies performed in ESCC have described various molecules that are differentially regulated as potential markers like pituitary tumor transforming gene (*PTTG*),⁸ transglutaminase 3 (*TGM*)⁹ and α actinin 4 (*ACTN4*).¹⁰ Some studies have focused on detecting presence of autoantibodies in ESCC patients and found antibodies in ESCC patient sera that were directed against CDC25B¹¹ and HSP70 among others.¹²

Many of the mass spectrometry-based proteomic studies have been employed to characterize cancer biomarkers.¹³ Labeling methods allow for multiplexing and relative quantitation of proteins based on the addition of chemical mass tags onto proteins. These labeling strategies have no effect on the analytical or biochemical properties of the labeled proteins or peptides. A number of chemical labeling strategies have been employed which include Isotope Coded Affinity Tags (ICAT) and Isobaric Tags for Relative and Absolute Quantification (iTRAQ). The iTRAQ method has been used as a quantitative approach for identification of biomarkers for a number of cancers including oral cancer,¹⁴ renal cell carcinoma,¹⁵ breast cancer,¹⁶ hepatocellular carcinoma,¹⁷ lung cancer,¹⁸ endometrial carcinoma¹⁹ and nasopharyngeal carcinoma.²⁰ Quantitative proteomics approaches have been used to study the ESCC proteome.²¹ However, in these studies, only a limited number of proteins were identified. An analysis of the literature clearly indicates that there is a need for more in-depth proteomics to explore the ESCC proteome further for biomarker discovery.

Here, we describe the use of an iTRAQ labeling strategy for quantitative proteomics using an Accurate Mass QTOF mass spectrometer to compare the protein expression profiles of pooled ESCC samples to pooled matched adjacent normal epithelium. Our study led to the identification of known as well as previously unreported molecules as candidate biomarkers for ESCC proving the utility of multiplexing methods such as iTRAQ for cancer biomarker discovery.

Results and Discussion

To identify candidate biomarkers for ESCC, a quantitative proteomic analysis was performed using iTRAQ-based in vitro labeling strategy followed by LC-MS/MS analysis. Esophageal squamous cell carcinoma tissue and matched adjacent normal epithelia from 10 patients were used in this study. The workflow employed in this study is shown in **Figure 1**. The details of the samples used in this study along with tumor grades are provided in **Table S1**.

iTRAQ labeling and LC-MS/MS analysis. Lysates were processed by labeling the adjacent normal epithelia-derived peptides

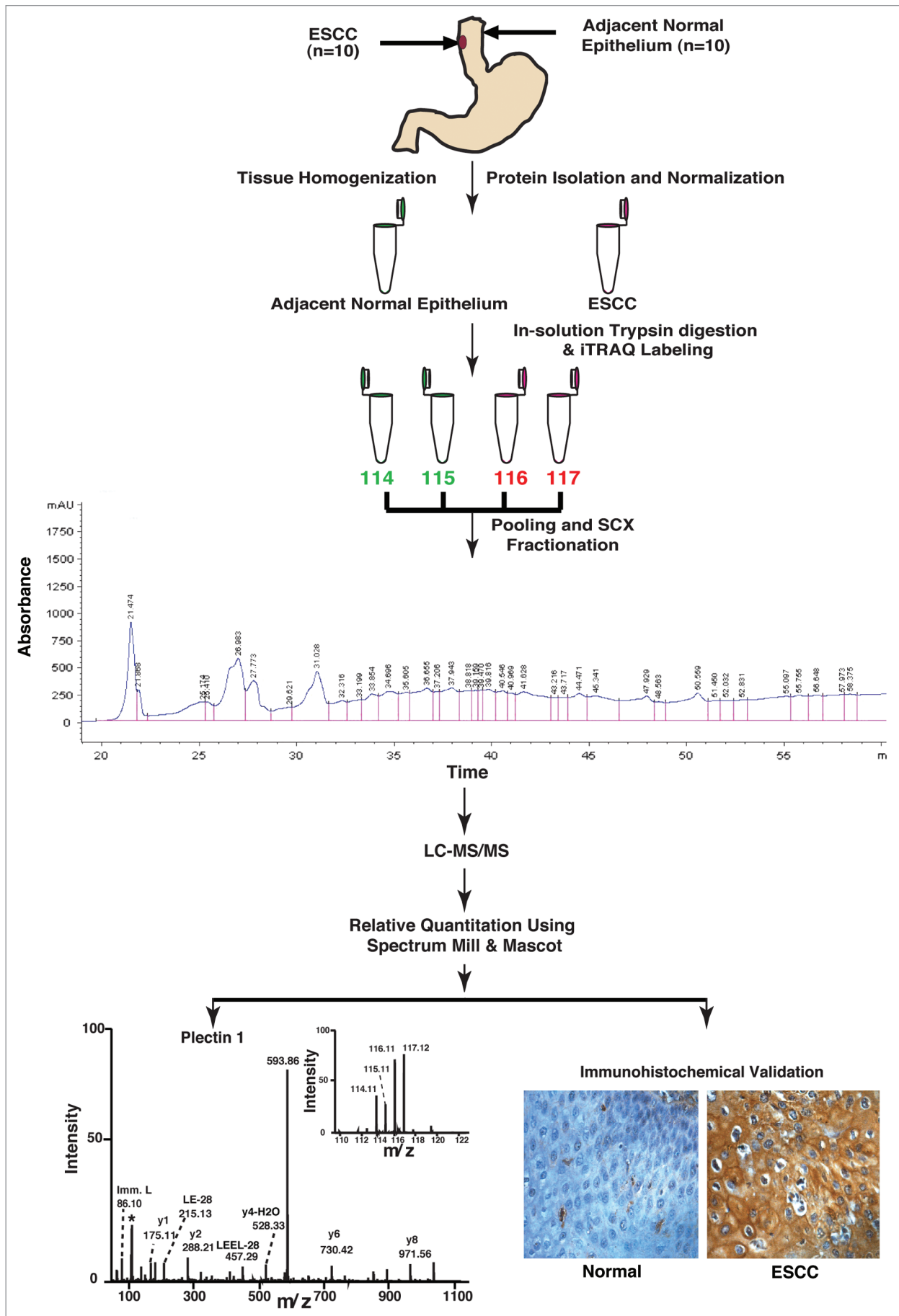
with 114 and 115 iTRAQ reagents and ESCC tissue-derived peptides with 116 and 117 iTRAQ reagents thus providing technical replicates within a single run. The data from a total of 41,151 MS/MS spectra generated by LC-MS/MS analysis of 157 SCX fractions were searched against the human RefSeq database using Spectrum Mill and Mascot search engines. An FDR cut off of 1% was applied to eliminate false positive identifications. This led to the identification of 687 proteins. A list of proteins identified in this study is provided in **Table S2**.

Quantitative analysis of LC-MS/MS data. Differentially expressed proteins in ESCC were quantified based on the iTRAQ ratios of peptides identified for these proteins. Quantitative analysis using Spectrum Mill and Mascot search engines led to the identification of 687 proteins. Of these, 147 proteins were upregulated >2-fold in tumor as compared with the adjacent normal epithelia, while 91 proteins were downregulated <2-fold in tumor. The list of the proteins with identified peptides using Spectrum Mill and Mascot are mentioned in **Tables S3 and S4** respectively.

Bioinformatics analysis of the data. Bioinformatics analysis was performed to classify proteins based on subcellular localization and biological function. We performed classification based on annotations in the Human Protein Reference Database (HPRD; www.hprd.org) in compliance with Gene Ontology (GO) standards. The summary includes fold-changes for differentially expressed proteins in ESCC. It also includes biological domains and motifs obtained from HPRD. The distribution of proteins identified in our study based on subcellular localization is shown in **Figure 2A** and according to biological processes is shown in **Figure 2B**. Among the 687 identified proteins, 206 contained signal peptides (SP), 26 contained a transmembrane (TM) domain and 13 contained both a TM domain and a SP motif. The MS and MS/MS spectra of representative known and novel proteins are represented in **Figure 3**.

Known overexpressed proteins identified in this study. Among the overexpressed proteins in ESCC tissues, we found number of proteins that had been previously described in ESCC, confirming the validity of the quantitative proteomic approach undertaken by us in this study. A partial list of known upregulated proteins is shown in **Table 1**. Among the proteins previously shown to be upregulated in ESCC were periostin (*POSTN*),^{22,23} thrombospondin 1 (*THBS1*) and fascin 1 (*FSCN1*). In one of the earlier studies on mRNA profiling of ESCC tissues, periostin was 11-fold upregulated in ESCC.²⁴ In the current quantitative tissue proteomic study periostin was 7-fold upregulated at the protein level in cancer tissue as compared with adjacent normal tissue.

Thrombospondin 1 is an extracellular adhesive glycoprotein that mediates cell-cell contact and is widely expressed. In the current study, thrombospondin 1 was 8-fold upregulated in cancer. Thrombospondin 1 has been previously shown to be overexpressed in ESCC and its overexpression correlated with regional lymph node invasion and poor survival of patients.²⁴ We have identified fascin 1 (*FSCN1*), an actin bundling protein, which plays diverse roles in cell-cell interaction, cell migration and is involved in increasing cell motility in transformed cells which was 2.5-fold upregulated in ESCC. In one of the previous studies,



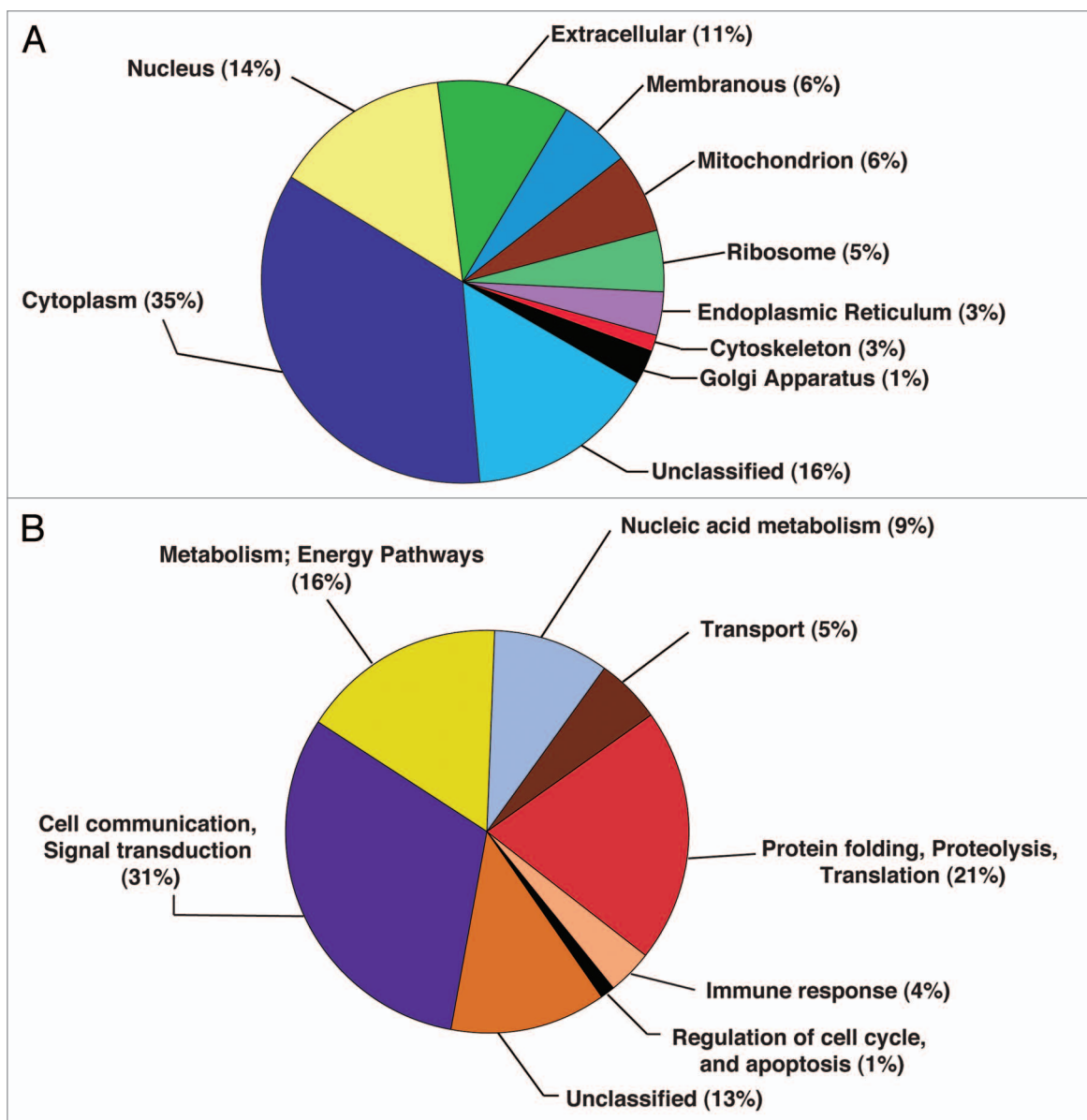


Figure 2. Classification of proteins by gene ontology based on their cellular localization and biological process. (A) Distribution of proteins based on their cellular component using gene ontology classifier. (B) Distribution of proteins based on their biological process using gene ontology classifier.

fascin 1 was shown to be upregulated in ESCC as compared with normal esophageal epithelia.²⁵ Stomatin-like protein 2 (*SLP2*) is a mitochondrial membrane protein involved in maintaining the mitochondrial membrane potential. Stomatin-like protein 2 affects cell motility and proliferation potentially by effecting the energy cycle within the cell, since it has a control on mitochondrial membrane potential. Stomatin-like protein 2 overexpression has been correlated with tumorigenesis and metastasis. In our study, Stomatin-like protein 2 was 3-fold upregulated in ESCC.

Wang et al. showed the correlation of Stomatin-like protein 2 downregulation with inhibition of proliferation and cell motility using wound healing assay and transwell assay in KYSE150 cell line. They also showed by IHC labeling that Stomatin-like protein 2 was overexpressed in ESCC tissues.²⁶

Fas associated via death domain (*FADD*) is a member of the TNF superfamily which mediates apoptotic signaling via its death domain. It is an adaptor protein that binds to Fas and TNF receptors upon ligand binding, thus mediating downstream

Figure 1 (See opposite page). Work flow for quantitative tissue proteomics using iTRAQ labeling and validation of biomarkers for esophageal squamous cell carcinoma. For iTRAQ labeling, proteins were isolated from ten tumor and adjacent normal tissues. Proteins were subjected to trypsin digestion followed by iTRAQ labeling of peptides. Post labeling of peptides the tumor and adjacent normal derived peptide mixture was pooled and fractionated using strong cation exchange (SCX) chromatography, followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) on a QTOF mass spectrometer. The data was searched using Mascot and Spectrum Mill search engines. Some of the overexpressed proteins that were not previously described (e.g., *PLECT*) were validated using IHC labeling using tissue microarrays.

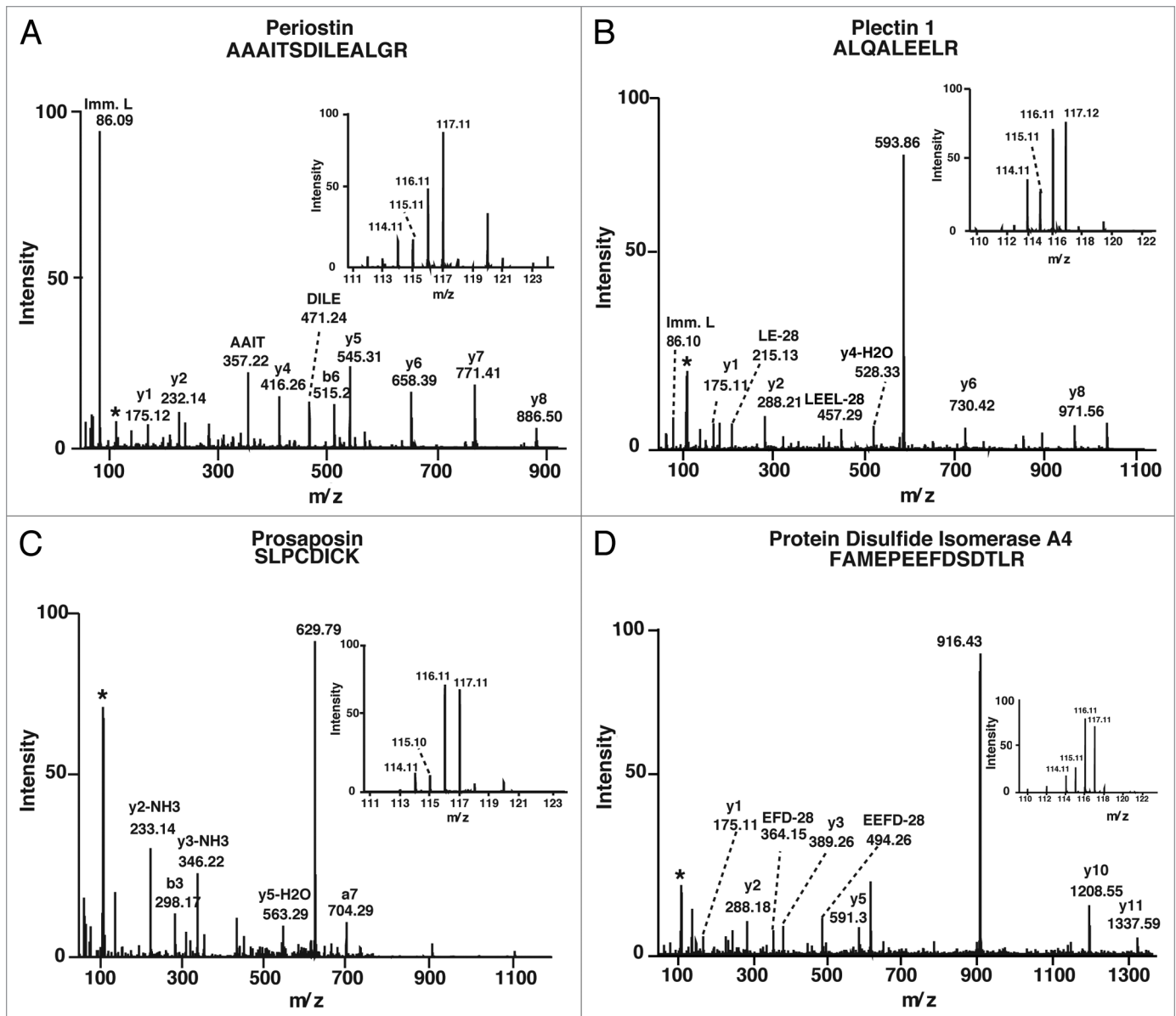


Figure 3. MS and MS/MS spectra of known and novel upregulated proteins in ESCC tissues as compared with the adjacent normal epithelia. MS and MS/MS spectra of peptide from representative differentially expressed proteins identified in this study. (A) Periostin (*POSTN*); (B) Plectin 1 (*PLEC1*); (C) Prosaposin (*PSAP*); and (D) Protein disulfide isomerase A4 (*PDIA4*).

apoptotic signaling. In a study by Xue et al. *FADD* was shown to be overexpressed in ESCC.²⁷ In our study, *FADD* was found to be 2-fold upregulated in ESCC tissue. Transgelin (*TAGLN*), also known as *SM22*, is an actin bundling protein which contains an actin binding region which is made of positively charged amino acids and CLIK domain (C-terminal calponin-like module). It is involved in binding actin filaments and in the process form podosomes. It is known that transgelin dysregulation results in cells becoming more malignant and invasive.²⁸ A proteomic study performed by Qi et al. showed that transgelin was upregulated in ESCC based on a comparative 2DE analysis of ESCC and normal esophageal tissue.²⁹ Our study is in concordance with study performed by Qi et al. as we also observed 2-fold upregulation of transgelin in ESCC.

Novel overexpressed proteins identified in this study. There was a subset of proteins that were observed to be upregulated, which have not been previously described in the context of ESCC. A partial list of these novel and upregulated proteins is shown in Table 2. Among these upregulated proteins, *PDIA4*, *PLEC1* and *PSAP*, have not been described in the context of ESCC. Cofilin 1 (*CFL1*) was found to be 3-fold upregulated in ESCC. Among other upregulated proteins in ESCC, ribophorin II (*RPN2*) was found to be 2-fold upregulated in our study. Ribophorin II is an ER membranous protein involved in N-glycosylation of newly synthesized proteins in ER. Overexpression of *RPN2* with respect to lymphovascular invasion is associated with poor survival in gastric cancer.³⁰ Carbonyl reductase 1 (*CBR1*) and carbonyl reductase 3 (*CBR3*) are cytoplasmic NADPH-dependent

Table 1. Partial list of overexpressed proteins that were previously reported in esophageal squamous cell carcinoma

S. no	Gene symbol	Protein	Biological features	Citation	Fold change upregulation in this study	
					Spectrum Mill	Mascot
1.	<i>THBS1</i>	Thrombospondin 1	It is a adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions.	Zhou, et al. (2009) ²⁴	8.5	8.2
2.	<i>POSTN</i>	Periostin	It is an extracellular matrix (ECM) protein found to be overexpressed in majority of the cases in epithelial and stromal compartments in ESCC. It is involved in EMT and possibly plays an important role in tumor invasion and metastasis.	Kashyap, et al. (2009) ²²	7.6	-
3.	<i>CTTN</i>	Cortactin	It is reported to be upregulated and overexpressed in early and late stages of ESCC. It is involved in organizing the cell adhesion complex.	Hsu, et al. (2009)	3.6	2.5
4.	<i>STOML2</i>	Stomatin like protein 2	An integral membrane protein involved in tumor progression. It has been shown to be overexpressed in ESCC.	Wang, et al. (2009) ²⁶	2.9	-
5.	<i>FSCN1</i>	Fascin homolog 1	An actin bundling protein involved in increasing cell motility in various transformed cells. It is overexpressed in ESCC.	Zhang, et al. (2006) ²⁵	2.5	2.8
6.	<i>FADD</i>	Fas-associated via death domain	Involved in death signaling initiated by TNF receptor. It was found to be overexpressed in ESCC and its corresponding precursor lesions.	Xue, et al. (2006)	2.7	-
7.	<i>CALR</i>	Calreticulin	An ER protein. It is a Ca(+2)-binding protein. It's overexpression was correlated with poor prognosis of ESCC patients.	Du, et al. (2009)	2.6	1.3
8.	<i>HSPA9</i>	Heat shock 70 kDa protein 9	<i>HSP70</i> overexpression has been reported in ESCC and the presence of autoantibodies against HSPA9.	Fujita, et al. (2008)	1.77	2.5

Table 2. Partial list of novel proteins identified as overexpressed in esophageal squamous cell carcinoma

S. no.	Gene symbol	Protein	Features	Fold upregulation	
				Spectrum Mill	Mascot
1.	<i>ILF3</i>	Interleukin enhancer binding factor 3	It is part of heterodimeric transcription factor NFAT (nuclear factor of activated T-cells). It is involved in mRNA binding, stabilization and in export of mRNA to cytoplasm.	2.17	2.36
2.	<i>CES1</i>	Carboxylesterase 1	It is involved in detoxification of xenobiotic and toxic compounds by hydrolysis. It is part of the drug metabolizing machinery in liver and other organs.	2.37	2.3
3.	<i>CFL1</i>	Cofilin 1	It is involved in polymerization and depolymerization of actin. It is shown to be overexpressed in non-small cell lung cancer (NSCLC) and overexpression of cofilin 1 was positively correlated with increased cellular invasiveness.	2.5	3.39
4.	<i>TAF15</i>	TBP-associated factor 15	It is part of the transcription machinery. It is involved in various activities like Promoter recognition and transcription initiation.	2.33	1.95
5.	<i>PPIB</i>	Peptidylprolyl isomerase B	It is endoplasmic reticulum (E.R.) resident chaperone involved in binding to cyclosporine A. It modulates the immunosuppressive action of cyclosporine. It also protects cells from E.R. stress induced cell death.	2.13	2.18
6.	<i>NCL</i>	Nucleolin	It is a nucleolus resident phosphoprotein involved in various processes like rRNA transcription, ribosome assembly and transport of ribosome components from nucleus to cytoplasm.	2.29	2.19
7.	<i>LYN</i>	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog	A protein kinase involved in erythroid differentiation. Reported as potential therapeutic target for breast cancer and involved in epithelial-mesenchymal transition (EMT) in breast cancer.	-	3.6
8.	<i>CBR1</i>	Carbonyl reductase 1	It catalyzes the reduction of many endogenous and xenobiotic carbonyl compounds. It is shown that during post-treatment of breast cancer cells with chemotherapeutic agents, expression of <i>CBR1</i> drastically increased possibly for detoxification purpose.	1.1	2.39

reductase which has been shown to be involved in reduction of a broad range of molecules including quinones, prostaglandins and antitumor agents. Carbonyl reductase 1 was shown to

be overexpressed in hepatocellular carcinoma under oxidative stress conditions like hypoxia.³¹ In the current study, carbonyl reductase 1 and carbonyl reductase 3 were 2-fold upregulated in

Table 3. Summary of IHC labeling for the validated molecules—*PLEC1*, *PSAP* and *PDIA4* in tumor and normal tissue

S. no.	Staining pattern	<i>PLEC1</i>	<i>PSAP</i>	<i>PDIA4</i>
1.	Positive	84	94	92
2.	Negative	16	6	8
3.	Strong	29	50	23
4.	Moderate	30	34	45
5.	Weak	25	10	24
6.	Normals stained	32	23	19
7.	p value	2.74E-013	9.59E-024	1.25E-024
8.	Subcellular location of staining	Cytoplasmic and membranous	Cytoplasmic	Cytoplasmic

ESCC. Cofilin 1 is an actin binding protein which is involved in polymerization and depolymerization of actin. It has been shown to be overexpressed in non-small cell lung cancer (NSCLC) and in this study overexpression of cofilin 1 was positively correlated with increased cellular invasiveness and resistance. Hence it was shown to be an effective prognostic and drug resistance marker.³²

Downregulated proteins in ESCC. There was a subset of proteins that were downregulated in the context of ESCC. 97 proteins were downregulated ≥ 2 -fold in ESCC tissue as compared with adjacent normal epithelia. Among the downregulated and known proteins, junction plakoglobin (*JUP*) was found to be 3-fold downregulated in ESCC as compared with normal. We have also identified Cystatin A (*CSTA*) and was found to be 3-fold downregulated in cancer. Osteoglycin (*OGN*) is a small protein belonging to the family of proteoglycans, which contains leucine rich repeats (LRR). It is involved in cellular growth, differentiation, cell growth and migration. It has been shown in many studies that downregulation of osteoglycin is related to increased metastasis.³³⁻³⁵ In the current study, osteoglycin was 3-fold downregulated in ESCC tissue as compared with adjacent normal epithelia.

Annexin 1 (*ANXA1*) is a calcium dependent phospholipid binding protein, phospholipids are precursor for arachidonic acid which are in turn used in biosynthesis of prostaglandins and leukotrienes known proinflammatory molecules. Annexin 1 is localized to the cytoplasmic face of the plasma membrane, where they bind to phospholipids upon stimulation and prevent access to phospholipase A2 (*PLA2G4A*). Annexin 1 is known to be downregulated in esophageal adenocarcinoma and squamous cell carcinomas,^{27,36-41} many studies have showed that *ANXA1* downregulation correlates with poorly differentiated status of oral squamous cell carcinoma cells. We also found that *ANXA1* was 3-fold downregulated in ESCC tissue.

Another molecule found to be downregulated in ESCC was cytokeratin 4 (*KRT4*), which is a basic cytokeratin usually present as a heterodimer with the acidic cytokeratin 13 (*KRT13*). It is expressed in differentiated layers of the mucosal and esophageal epithelia and has been shown to be downregulated during progression of ESCC. Xue et al. analyzed cytokeratin 4 in a panel of proteins using tissue microarrays of different stages of tumors varying from precursor lesions to metastasis.²⁷ Downregulation of cytokeratin 4 was observed during progression from dysplasia and carcinoma in situ to metastasis. In our study, cytokeratin 4

was 9-fold downregulated in ESCC. Lastly, our quantitative proteomics study provides validation for several molecules at the protein level that were reported earlier at the mRNA level and in concordance with current study, including, cystatin A (*CSTA*) and small proline-rich protein 3 (*SPRR3*).³⁸

Validation of novel candidate biomarkers by immunohistochemical staining. Some of the candidate proteins identified in our study were further tested by IHC labeling to assess whether they could serve as potential biomarkers for ESCC. The selection of proteins was based on the extent of overexpression, biological importance, involvement in other tumors and availability of commercial antibodies suitable for IHC labeling. We selected plectin 1 (*PLEC1*), prosaposin (*PSAP*) and protein disulfide isomerase 4 (*PDIA4*) for further validation in formalin fixed paraffin embedded tissue sections. All of these proteins were overexpressed at the protein level with ≥ 2 -fold change in ESCC as compared with the adjacent normal epithelium (see Table 2), and were validated using IHC labeling. A statistical analysis was performed to determine the significance of differential expression of *PDIA4*, *PSAP* and *PLEC1* between ESCC and normal tissues using a Chi-Square test. There was a statistically significant ($p < 0.05$) difference in the expression of *PDIA4*, *PLEC1* and *PSAP* between tumor and normal controls. The IHC staining pattern of these molecules in tumor and normal tissues is summarized in Table 3. The IHC scores for all the ESCC patients for plectin 1, prosaposin and protein disulfide isomerase A 4 are provided in Table S5.

Plectin 1 (*PLEC1*). Plectin 1 (*PLEC1*) is the member of the plakin family. It is a multi-domain protein involved in crosslinking different cytoskeletal proteins and plays an important role in maintaining cell architecture and shape.⁴² Plectin was originally isolated as an intermediate filament binding protein. It is localized in the basal layer of epidermal cells where it appears to be a component of both hemidesmosomes and desmosomes.^{43,44} Plectin is also involved in connecting cytokeratins to $\alpha\beta 4$ integrin at hemidesmosomes. It has been shown that *PLEC1* is involved in microfilament network reorganization during apoptosis since caspase-8 cleaves *PLEC1* upon activation during early stages of apoptosis. However this cytoskeletal remodeling is disturbed in cancer cells since most apoptotic pathways are dysregulated in cancer cells leading to accumulation of *PLEC1*. In one study on pancreatic intraductal papillary mucinous neoplasms (IPMN), it was shown that *PLEC1* was upregulated in malignant

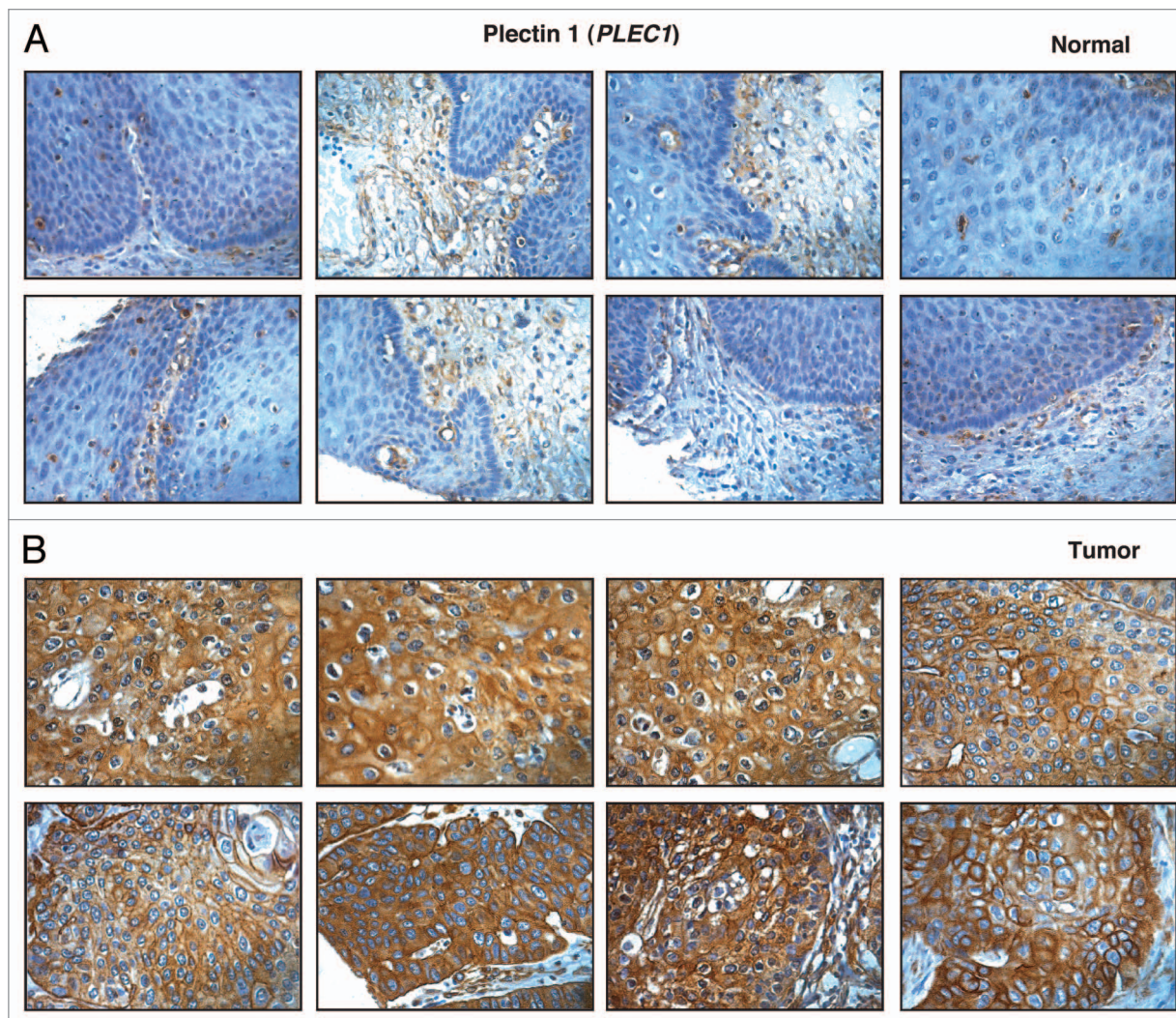


Figure 4. Validation of Plectin 1 using immunohistochemical labeling. Representative sections from tissue microarrays stained with anti-Plectin 1 is shown (A) expression of Plectin 1 in representative normal esophageal squamous mucosa and (B) expression of plectin 1 in ESCC.

IPMNs.⁴⁵ In our study, *PLEC1* was 2-fold upregulated in ESCC tissue. Immunohistochemical labeling for *PLEC1* showed overexpression of *PLEC1* in 84/100 ESCC cases and expression in the majority of cases was cytoplasmic and membranous. The staining pattern of *PLEC1* in representative ESCC and normal esophageal tissue is shown in **Figure 4**.

Prosaposin (PSAP). Prosaposin is a lysosomal compartmental protein involved in catabolism of sphingolipids with small sugar chains. It is 526 amino acids long with a molecular weight of 58 kDa and is present as both membrane and secreted forms in the lysosome. It facilitates degradation of sphingolipids by making the lipid substrate more accessible to the soluble degradative enzymes in the lumen of lysosome.⁴⁶ Prosaposin is a precursor protein which is partially cleaved into structurally related activator proteins saposin A, B, C and D. These saposins are known to play different biological roles. Prosaposin is a known neurotrophic factor⁴⁷ and prosaposin deficiency or deletion is lethal in human and mice. It has been shown that prosaposin is amplified and overexpressed in number of androgen independent human

prostate cancer cell lines (metastatic cell lines-PC-3, DU-145, MDA-PCa 2b, M-12, NCI-H660).^{48,49} In some of the early studies *PSAP* has been shown to prevent apoptosis and promote survival in prostate cancer cells⁵⁰ and *PSAP* is shown to upregulate androgen receptor (AR), prostate specific antigen (PSA) in prostate cancer cells (LNCaP cells).⁵¹ In the process, it acts as androgen-agonist and its growth promoting effect may give a selective growth advantage to these prostate cancer cells, hence in the process it acts as an androgen regulated gene. In our study, *PSAP* was 4-fold upregulated in ESCC tissue. Immunohistochemical labeling for *PSAP* showed overexpression of *PSAP* in 94/100 ESCC cases and expression in the majority of cases was cytoplasmic. The staining pattern of *PSAP* in representative ESCC and normal esophageal tissue is shown in **Figure 5**.

Protein disulfide isomerase 4 (PDIA4). Protein disulfide isomerase 4 is an enzyme resident in the endoplasmic reticulum which is a thiol isomerase involved in facilitating the formation, reduction and rearrangement of disulfide bonds in nascent proteins. The extent of sequence identity between different members

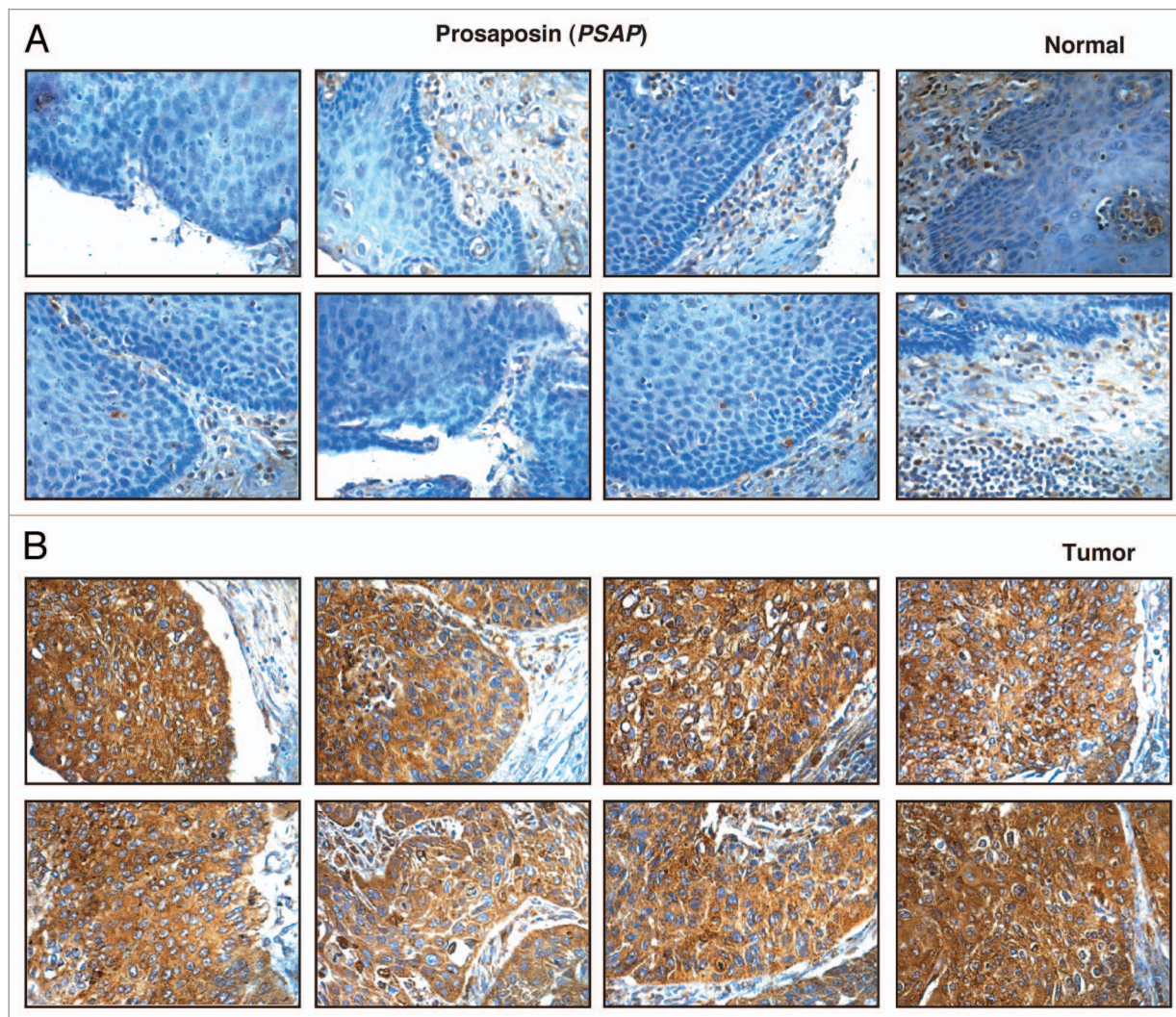


Figure 5. Validation of Prosaposin using immunohistochemical labeling. Representative sections from tissue microarrays stained with anti-prosaposin is shown (A) expression of prosaposin in representative normal esophageal squamous mucosa and (B) expression of prosaposin in ESCC.

of PDI family is relatively low; however they share active-site motifs and domain structure. *PDIA4* is 645 amino acids long and has a molecular weight of 72 kDa; thus it is also known as *ERP72*. Most protein disulfide isomerases have three conserved domains—A, B and C.^{52,53} Domain A is a catalytically active thioredoxin like domain, domain B is a catalytically inactive thioredoxin like domain and domain C is a highly acidic region which is frequently used to bind to peptides.⁵⁴ *PDIA4* differs from other PDIA family members other in having 3 active thioredoxin domains rather than two with the domain distribution being C-A-A-B-B-A. Like other PDIA family members, *PDIA4* is a stress induced protein and hence it is seen in many different cancers especially in tumor induced hypoxic states. In one of our earlier studies where we analyzed the secretome from ESCC and normal esophageal cell lines, *PDIA4* along with *PDIA3* was shown to be upregulated in the secretome of ESCC cell lines as compared with normal esophageal cell line.⁵⁵ In this study, *PDIA4* was again observed to be 2-fold upregulated in ESCC tissue. Immunohistochemical labeling for *PDIA4* showed

overexpression of *PDIA4* in 92/100 ESCC cases and expression in the majority of cases was cytoplasmic. The staining pattern of *PDIA4* in representative ESCC and normal esophageal tissue is shown in Figure 6.

Public availability and accessibility of proteomic data. To make our observations publicly available and accessible to other researchers, we have submitted our data on the immunohistochemical analysis and the list of proteins and peptides identified to Human Proteinpedia (HUPA, www.humanproteinpedia.org).⁵⁶ The raw data described in this study is freely available from ProteomeCommons.org. Online versions of the data may be found at www.proteomecommons.org/dataset.jsp?i=75,354 from the proteomecommons.org main page or alternatively it can be located in the Tranche data repository and can be downloaded through the Tranche server Tranche Hash. Data Set 1-“bxX7cPEuBv9wUkgqq4uCRwWqc415/tGX+X5x6BNFZRu9tK3ZXhDqZwvOVzWFeChRFIh3O2t7sxpZsNF1YULs/sEmcoAAAAAAL3Zg==,” Data Set 2-“KNSTIp1uYntNYp9n9NWDKh5zeUICcmSapzEL467Tiiys

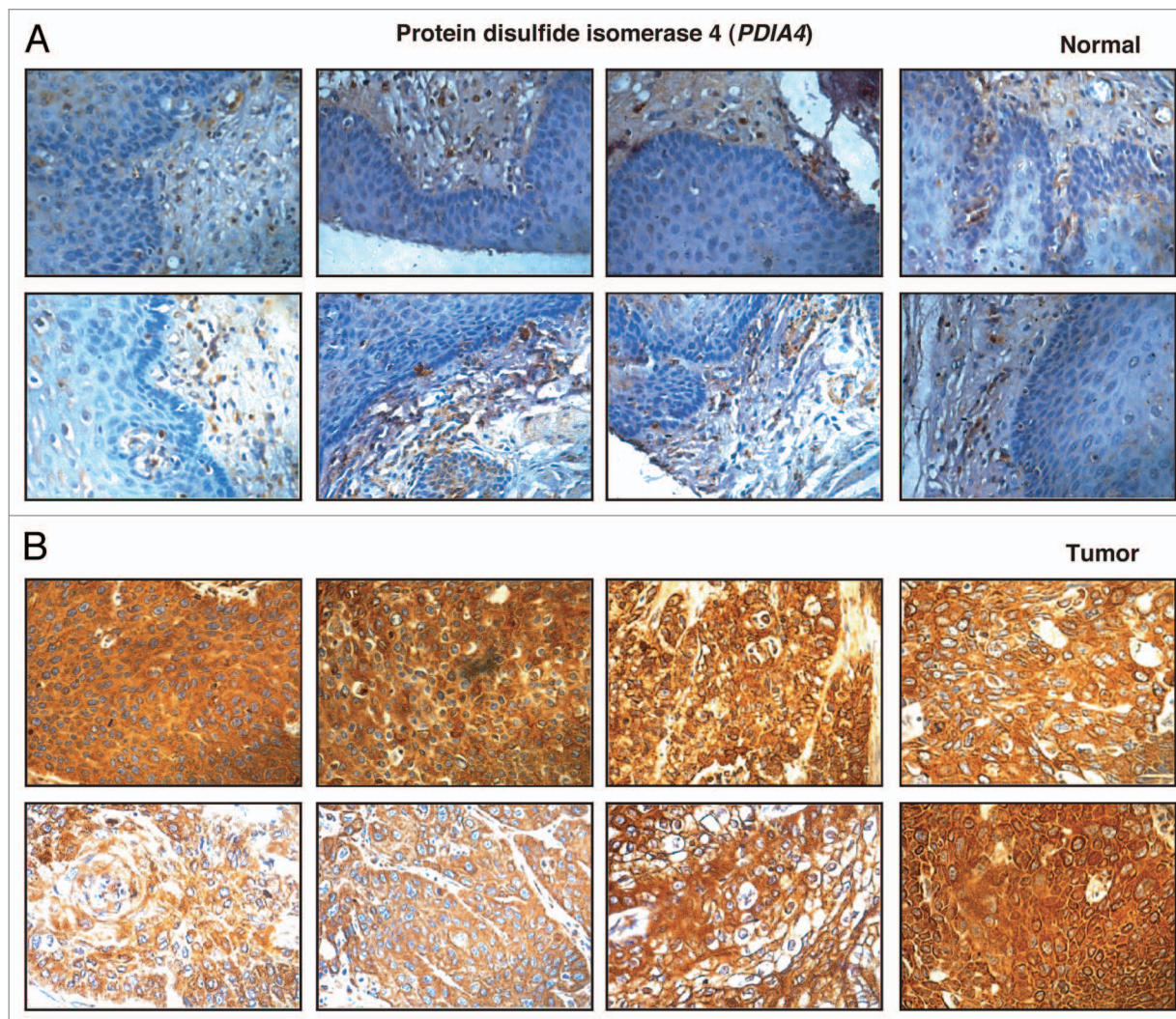


Figure 6. Validation of protein disulfide isomerase A4 using immunohistochemical labeling. Representative sections from tissue microarrays stained with anti-protein disulfide isomerase A4 as shown (A) expression of protein disulfide isomerase A4 in representative normal esophageal squamous mucosa and (B) expression of protein disulfide isomerase A4 in ESCC.

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Materials and Methods

Tissue collection and storage. This study was approved by the institutional review board (IRB) at the Kidwai Memorial Institute of Oncology, Bangalore. The tumor tissues with their adjacent normal epithelium were obtained after surgical resection. All patients were classified as T3N1M0 with the histopathological grade ranging from well to poorly differentiated ESCCs. Informed consent was obtained from all subjects enrolled in the study prior to collection of tissue samples. The normal tissue was at least 5 cm away from the margins of ESCC. All specimens were histologically confirmed by an expert pathologist (RVK).

Protein isolation and iTRAQ labeling. In this study, we used ESCC tumor tissues samples from ten patients along with the corresponding adjacent normal epithelia. Tissue homogenization and lysate preparation was performed as previously described in reference 17. Briefly, 10 mg of esophageal tissue was homogenized in 0.5% SDS using cell disperser (IKA works, Wilmington, NC) followed by sonication. The cell debris from the tissue homogenates was removed by centrifugation at 14,000 rpm for 30 min at 4°C. The cleared supernatant was transferred into a microfuge tube and the protein concentration determined using the Lowry assay. Equal amounts of lysates (100 µg) from normal and cancer tissue samples were treated with 2 µl of reducing agent [tris(2-carboxyethyl) phosphine (TCEP)] at 60°C for 1 h and alkylated with cysteine blocking reagent, methyl methanethiosulfonate (MMTS) for 10 min at room temperature. The samples were digested overnight with sequencing grade trypsin (Promega, Madison, WI) (1:20) at 37°C and peptides labeled with iTRAQ reagents as

follows: pooled adjacent normal tissue derived peptides were labeled with reagents containing reporter ions of m/z 114 and 115; while those from pooled ESCC tumor tissue were labeled with reagents containing reporter ions of m/z 116 and 117. The labeled samples were pooled and mixed with Solvent A (10 mM KH_2PO_4 , 20% acetonitrile, pH 2.8). The diluted samples were manually injected onto a 200 μl bed volume of a strong cation exchange (SCX) column (PolyLC Inc.). Peptides were loaded with a flow rate of 200 μl per min and subsequently washed for 20 min. A gradient of 30 min from 8% Solvent B (350 mM KCl in solvent A) to 50% Solvent B was used for fractionation. Fractions collected were dried and stored at -20°C until LC-MS/MS analysis.

LC-MS/MS analysis. iTRAQ labeled peptides after SCX fractionation were analyzed using the 6520 Accurate Mass QTOF mass spectrometer (Agilent Technologies, Santa Clara, California, USA) interfaced with an HPLC Chip system (Agilent Technologies, Santa Clara, CA). We used the HPLC Chip system comprising of a 40 nl enrichment column and a 43 mm x 75 μm analytical column, both made up of a reversed-phase material Zorbax 300SB- C_{18} with a particle size of 5 μm . The samples were loaded on the enrichment column using Agilent 1200 series capillary liquid chromatography system equipped with a micro-well plate autosampler at a flow rate of 3 $\mu\text{l}/\text{min}$ using solvent A (0.1% formic acid). An injection flush volume of 4 μl was applied during the enrichment step. The peptides were eluted from the analytical column at the flow rate of 400 nl/min using a gradient of 3–40% solvent B (90% Acetonitrile, 0.1% formic acid) over 30 min. Data dependent acquisition was performed using MassHunter data acquisition software in auto MS/MS mode with precursor survey scans for 1 sec (from 350–1,800 m/z) followed by three MS/MS scans at a rate of 3 spectra/s (50–2,000 m/z). Three most intense ions were chosen for fragmentation with charge state preference in the order of +2, +3 and +4. Dynamic exclusion was set for 2 MS/MS scans of the same precursor ion and released after 2 min.

Data analysis. The mass spectrometry data was searched using Spectrum Mill (Agilent Technologies, Rev. A.03.03.) and Mascot (Matrix Science Inc., Version 2.2.0). MS/MS data were processed to generate mascot generic format (mgf) files and searched against human RefSeq Build 35 protein sequence database (34,906 sequences) by selecting oxidation of methionine, iTRAQ 4-plex modification at peptide N-terminus and lysine (K) as variable modifications and carbamidomethylation of cysteine as a fixed modification. MS and MS/MS tolerance were set to 100 ppm and 0.1 Da, respectively. Two missed cleavages were allowed. False discovery rate (FDR) was calculated by a decoy database search strategy. Peptide spectrum matches (PSMs) at 1% FDR were used for protein identifications. Relative protein quantitation was performed using Spectrum Mill and Mascot.

Bioinformatics analysis. Proteins were classified into groups based on their primary subcellular localization, biological process and molecular function. These analyses were performed through the Human Protein Reference Database (HPRD)⁵⁷

which complies with GO terminologies for functional description of proteins.

Antibodies. Immunohistochemical staining was performed on formalin fixed paraffin-embedded sections using different antibodies. Of the three antibodies used, one was rabbit monoclonal (anti-*PLECI*) and the other two were rabbit polyclonal antibodies. The anti-*PDIA4* antibody was used at a dilution of 1:20 (catalog # HPA006140, Human Protein Atlas, Stockholm, Sweden), anti-*PSAP* antibody at a dilution of 1:250 (catalog # sc-32875, Santa Cruz Biotechnology Inc., Santa Cruz, CA) and anti-*PLECI* antibody at a dilution of 1:100 (catalog # ab32528, Abcam, Cambridge, MA).

Immunohistochemical labeling for validation. Archived paraffin embedded tissue sections of ESCC samples were obtained from the Department of Pathology, Kidwai Memorial Institute of Oncology, Bangalore. Validation of potential biomarker was performed on a large number of samples using tissue microarrays (TMAs) (n = 100). Tissue microarrays for formalin fixed ESCC from different sources were also used. Commercially available tissue microarrays were obtained from Creative Bioarray (New York) consisting of 60 ESCCs with matched normal esophageal epithelia. The ESCCs varied from tumor grade I to grade III from patients in the age group of 39–79 y. A second group of tissue microarrays obtained from FolioBio (Columbus, OH) consisted of 40 ESCC tissues with matched adjacent normal esophageal squamous epithelium. The ESCC tissues varied from tumor grade I to III from patients in the age group of 43–76 y. The IHC staining was performed as described previously in reference 58. The TMAs were deparaffinized by incubating the slides at 58°C for 2 h. The statistical significance of differential staining was determined using a Chi-square test. Results for expression of *PSAP*, *PLECI* and *PDIA4* were considered statistically significant if the p value was < 0.05 . The statistical analysis was performed using R version R-2.13. The scoring of immunohistochemical staining of tissue sections was performed as previously in reference 55.

Conclusions

This is the most extensive quantitative proteomic study in ESCC performed to date. Using an iTRAQ-based quantitative proteomic approach to compare ESCC tissue with its adjacent normal epithelium, we have identified 687 proteins from ESCC tissue which include several proteins reported in previous studies emphasizing their overexpression. We also report many novel proteins which were not previously reported by any other study to be associated with ESCC. The immunohistochemical labeling for *PDIA4*, *PSAP* and *PLECI* further support the potential of these molecules to be pursued as biomarkers. Their overexpression in ESCC tissue proteome could lead to overexpression in blood and body fluids of the affected patients. Further testing will be necessary to assess them as biomarkers for early detection of ESCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental materials can be found at: www.landesbioscience.com/journals/cbt/article/16833

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