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Comparative proteome analysis of human esophageal cancer and adjacent normal tissues

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ABSTRACT

Objective(s): Ranking as the sixth commonest cancer, esophageal squamous cell carcinoma (ESCC) represents one of the leading causes of cancer death worldwide. One of the main reasons for the low survival of patients with esophageal cancer is its late diagnosis.
Materials and Methods: We used proteomics approach to analyze ESCC tissues with the aim of a better understanding of the malignant mechanism and searching candidate protein biomarkers for early diagnosis of esophageal cancer. The differential protein expression between cancerous and normal esophageal tissues was investigated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Then proteins were identified by matrix-assisted laser desorption/ ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) and MASCOT web based search engine.
Results: We reported 4 differentially expressed proteins involved in the pathological process of esophageal cancer, such as annexinA1 (ANXA1), peroxiredoxin-2 (PRDX2), transgelin (TAGLN) andactin-aortic smooth muscle (ACTA2).
Conclusion: In this report we have introduced new potential biomarker (ACTA2). Moreover, our data confirmed some already known markers for EC in our region.

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Introduction

Esophageal cancer (EC) is the sixth main cause of cancer death around the world. Patients diagnosed with progressive form of esophageal cancer have low chance of recovery (1). Esophageal cancer can be divided into two major histologic subtypes including esophageal squamous cell carcinoma (ESCC), the most prevalent one, and esophageal adenocarcinoma (EAC)(2). Both genetic susceptibility and environmental risk factors are involved in the initiation of EC. The variation in the international incidence rates of esophageal cancer is about 16-fold in the highest-risk area called "esophageal cancer belt" which extends from northern Iran to north-central China where 90% of ESCC cases are reported (2).

A great number of diagnosed EC patients have progressive metastasis and are not good candidates for surgery (2) which is due to the late diagnosis. Thus, the detection of cancer in early stages is of utmost importance for the therapeutic management of EC (3). Over the past years, the molecular etiology of esophageal cancer has been studied extensively in both gene expression and protein expression levels in an attempt to find target biomarkers for the development of detection and therapeutic strategies. Proteomics methods have been used in an attempt to study differentially expressed proteins in cancer cells compared with normal tissues. Two-dimensional electrophoresis is a well-known technique used for proteomics analysis of numerous human cancers such

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as colorectal, gastric, breast, and lung, prostate and pancreatic cancer (4-9). The main aim of cancer proteomics is to recognize biomarkers and to improve clinical outcomes. Identification of biomarkers plays an important role in diagnosis of cancer in early stage. Moreover, it may be also helpful in the recognition of new targets involved in tumorigenesis (10). Although previous proteomics studies in EC have identified some proteins with differential expression in esophageal tumors, but the heterogeneity of esophageal tumors as well as diversity of biological mechanisms highlights the need for new researches (11, 12). Although, Iran is one of the countries with high prevalence of EC with most of cases located in the north of the country (11) but there are only two proteomics reports for esophageal cancer in Iran (13, 14). In the current study, total of 3 human EC specimens were collected during tumor surgical resection in Omid Hospital, Mashhad, Iran. We employed a patient-based proteomic approach and MALDI-TOF/TOF mass spectrometry to compare and to analyze significantly different expressed proteins in EC and adjacent non-tumor tissue samples. These finding may be useful in identifying biomarkers involved in EC.

Materials and Methods

Reagents

IPG strips (pH 3–10, 17 cm), Bio-Lyte (pH 3–10), proteinassay kit were purchased from BioRad (USA). Acrylamide, SDS, urea, thiourea, Tris-HCl, and glycine were obtained from Merck (Germany). CHAPS, DTT, and iodoacetamide were purchased from Sigma-Aldrich. Ultrapure agarose was purchased from Invitrogen (USA).

Patients and tissue samples

A total of 3 human EC specimens were used. These samples were collected during tumor surgical resection in Omid Hospital, Mashhad, Iran. All tumor samples were obtained with patient consent, without identifiers. After surgery, all tissue specimens were examined pathologically to obtain representative, viable, and non-necrotic tissues. All three patients were in the same stage. The esophageal cancer tissues were pathologically identified as representative, non-necrotic tissues carcinoma. These specimens were frozen at -80 ℃ and sectioned into thick sections. The adjacent normal tissue of each patient used as a control.

Sample preparation

First, tissue samples (200 mg) were washed three times with PBS to remove residual blood. The dissected normal and malignant tissues were homogenized in rehydration buffer (6 M urea, 2 M thiourea, 2% CHAPS, 50 mM DTT, and 0.2% Bio-lyte and complete protease inhibitor cocktail (Sigma) using Polytron Homogenizer (IKA®T10, Germany) and sonicated on ice using a probe sonicator (UP100H, Germany), then centrifuged at 14000 g. Total protein concentrations of supernatants were measured using Bradford protein assay kit.

Two-dimensional electrophoresis

First-dimension, isoelectric focusing (IEF), was carried out on a Protean IEF cell (BioRad). 150 µg of total protein to a final volume of 300 µl, was loaded onto a 17 cm nonlinear pH gradient (IPG) strips (pH 3-10), and was allowed to get actively rehydrated for 12 hr at 50 V using protean IEF cell (Bio-Rad, USA). Rehydrated IPG strips, then, were focused according to following protocol in 4 steps: 250 V for 15 min; 250-8000 V for 2.5 hr; 8000 V for 11 hr until 60,000 Vh; 500 V. Following IEF, IPG strips were immediately incubated in equilibration buffer (Tris–HCl (37.5 mM, pH 8.8), urea (6 M), glycerol (30%, v/v), SDS (2%, w/v), and DTT (1%, w/v)) on a rocking bed for 1 5 min, then strips were incubated in the same equilibration buffer (without DTT) containing iodoacetamide (2.5%, w/v) while rocking for 15 min. For the second dimension, electrophoresis was performed using 12% SDS-poly acrylamide gels. Briefly, the equilibrated IPG strips were placed on top of a SDS-PAGE and sealed with agarose (0.5%, w/v). Gels were run in Tris-glycine running buffer (pH 8.3) using POWER/PAC 3000 (BioRad) at 120 V for 5 hr, and then at 150 V until the bromophenol blue dye reached to the bottom of the gel. For colloidal Coomassie blue staining, gels were rinsed 5 times with distilled water for 10 min, then the gels were stained with 0.02%,w/v colloidal Coomassie solution containing G-250 (5%, w/v), aluminum sulfate-(14-18)-hydrate, ethanol (10%, v/v), orthophosphoric acid (2%, v/v), while rocking for 12 hr.

Gel image analysis

Protein patterns in the stained 2D gels were recorded using an Image Scanner III, Lab Scan 6 (Epson J181A, Japan). Spot detection and image matching were performed with Image Master Platinum 6.0 software (GE Healthcare, USA).For protein matching, protein gel maps of normal controls were considered as a reference and the analysis of differential expression was performed by software. Spots that were either present in only one of the groups or showed statistically significant (P<0.05) changes in expression were selected for identification by MALDI-TOF/TOF.

In gel digestion, MALDI-TOF-MS identification of peptide mixtures and database searching

Protein spots displaying change more than 1.5 and less than 0.6 fold were selected for protein identification. These spots were cut out and transferred to a microtube containing 1% acid acetic and sent to Center of Genomic Sciences at University of Hong Kong for in gel digestion and mass analysis using MALDI-TOF/TOF (Matrix Assisted Laser

Desorption Ionization-Time of Flight Analyzer) mass spectro-metry (ABSciex, Framingham, MA). Mass data were submitted to web-based MASCOT software (Version 2.1.0, Matrix Science, London, UK). MASCOT search parameters were calibrated as follows: taxonomy: Homo sapiens; peptide charge: +1, monoisotopic; MS/MS fragment tolerance: ± 0.2 Da; precursor mass tolerance: 75 ppm; cysteine carbamidomethylation for fixed modification; methionine oxidation for variable modification. Proteins with confidence intervals of 100% were accepted.

Classifications and network interaction analysis

For classification of altered proteins, PANTHER (protein analysis through evolutionary relation-ships) online database (http://www.pantherdb.org) was used. We utilized this website to categorize identified proteins according to their biological process, molecular function and biological pathways (15).

Statistics

Statistical analysis was performed using the SPSS version 16.0. Comparisons of mean values between normal and cancerous samples were made by T test.

Results

The proteome profiles of tumor were compared to that of in normal groups.

Analysis of the images by software showed that four spots have significant differences in the expression (P<0.05) (Figure. 1).

These four differentially expressed protein spots in the EC tumor and adjacent nontumor tissue were identified by MALDI-TOF-TOF and MASCOT search engine.

These proteins were annexinA1 (ANXA1), peroxiredoxin-2 (PRDX2), transgelin (TAGLN) and actin-aortic smooth muscle (ACTA2).

Among altered proteins, levels of three proteins, (ANXA1, PRDX2, TAGLN) significantly decreased, while the level of ACTA2 considerably increased in the EC tumor as compared with adjacent non-tumor tissue.

The information of identified proteins including protein name/gene names, size, Swiss-Prot accession number, theoretical pI, fold change, confidence interval(C.I.%), protein score, sequence coverage (%), sequence of identified peptide with the highest ion score and biological function are listed in Table 1.

Classification of differential protein expression

To classify proteins according to biological function, their gene name was submitted to PANTHER database (Figure 2). The proteins were classified into seven classes.

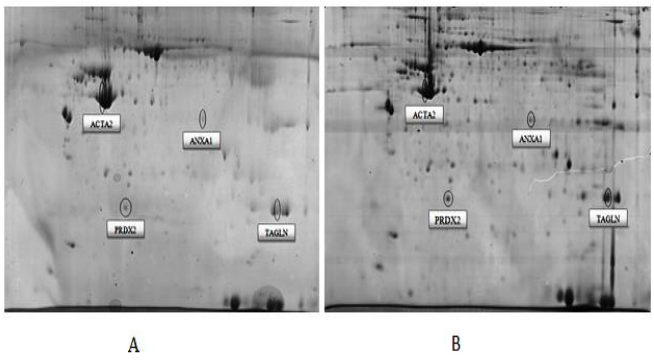

Figure 1. Two-dimensional gel electrophoresis of EC samples (A) and normal tissue (B)

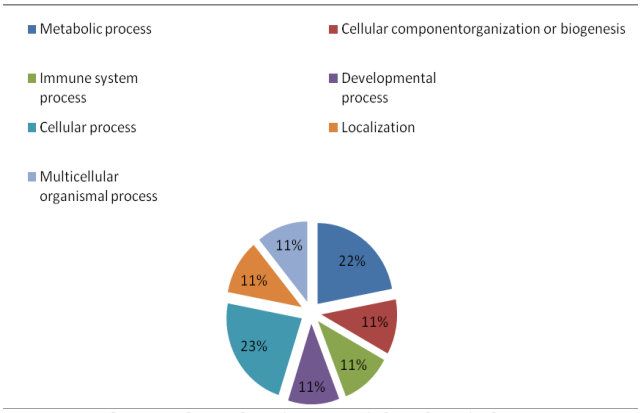

Figure 2. The pie chart classification of the identified proteins according to their biological process

Discussion

In this paper, we employed comparative proteome study between normal and cancerous esophageal tissues to identify potential diagnostic biomarkers that could be used in disease detection and monitoring. The expression levels of three proteins decreased while one protein increased in cancerous tissue of all three independent paired samples.

The identified proteins including annexinA1 (ANXA1), peroxiredoxin-2 (PRDX2), transgelin (TAGLN) and actin-aortic smooth muscle (ACTA2) are categorized as antioxidant, binding, catalytic, and structural molecule. The interaction between these proteins showed in Figure. 3.

The reduction in protein levels of ANXA1, TAGLN and PRDX2 was 60%, 48% and 45%, respectively, while actin level was elevated by 70% in EC tissue as compared to normal tissue. ANXA1 or lipocortin 1, is a calcium-phospholipid-binding protein involved in anti-inflammatory, anti-proliferative and apoptosis process (16, 17).

It has been reported that changes in the expression of Annexin superfamily members linked with different aspects of cancer, including angiogenesis, signal transduction, tumor invasion, metastasis and apoptosis.

The expression of annexins increased in some types of cancer while it was down-regulated in others (18-20). Down regulation of annexin A1 has been shown in several cancers such as esophageal, prostate, gastric, and head and neck (21 -25). ANXA1 can reduce cell proliferation through the regulation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signal transduction pathway (26, 27).

This protein has also a role in development and progression of other carcinomas such as breast cancer (28). There are some discrepancies among the published results about annexin 1 expression level in esophageal cancer. While some reported the overexpression of annexin1 but reduced expression of annexin1 has also been reported by others (14, 29, 30). Low expression of ANXA1 was reported to be associated with early onset of tumorigenesis in EC (11). It seems that these discrepancies may be due to the multistage characteristics of esophageal cancer. For example, the results reported by Wang *et al* revealed that there was a correlation between the high level of cytoplasmic annexin 1 and high pathologic stage in esophageal carcinoma and it may have a promoting role on the progression of esophageal cancer due to its connection with EGFR (31). TAGLN, an actin stress fiber-associated protein is mainly located in the cytoplasm of normal smooth muscle of adult vertebrates. Disorganization of the actin cytoskeleton is a fundamental event of the developing cancer cell phenotype. TAGLN expression is down-regulated in many cell lines, and this may be an early marker of the onset of transformation and may act as a tumor suppressor (32). It has been shown that expression of TAGLN is decreased in prostate, breast and colon cancers (33, 34). There are some controversies on the overexpression or down-regulation of TAGLN in ESCC. While the results of two separated proteomics studies showed increased expression level of TAGLN (35), other proteomics studies for finding biomarkers among tumor-associated proteins reported down regulation of TAGLN in ESCC patients (36, 37).

Our results are in agreement with previous studies where a reduction in the expression of TAGLN in esophageal cancer tissues was detected. There are some possible reasons for these contradictory observations.

The expression and biological function of TAGLN in cancer is related to both cancer type and stage of the tumor and may change during tumor progression (38).

PRDX2 is a member of antioxidant enzyme family of PRDXs (40). It has been recently showed that beside the known antioxidant activity, it is involved in self-defense against infection, tissue damage and tumors through its regulatory effect on inflammation (41).

Aberrant expression of PRDXs has been shown in different types of cancer. PRDX2 is down expressed in melanoma (42) and bladder cancer (43) and over expressed in breast cancer (44). PRDX2 protects cell from oxidative damage and protects cancer cells from DNA-damaging agents (45). There is a low overlap among the previous proteomics studies in esophageal cancer belt countries including China (36, 39, 46-49), Japan (50, 51) India (37) and, Iran (14).

This can be due to the differences among methods used by each group (2) and some known distinct variations in esophageal cancer geographic region, race, ethnicity and molecular epidemiology (36, 52).Our study revealed that ACTA2 was up regulated in tumor samples. The result of Fu *et al* (2007) revealed that ACTA4 has increased in ESCC specimens (47) but over expression of ACTA2 was reported for the first time in our present study. Actin filaments are involved in fundamental cellular communication and cell motility processes (54) and ACTA2 is essential for metastasis in lung adenocarcinomas and can be used as a promising prognostic biomarker.

The influence of ACTA2 on the dynamics of cytoskeletal structures is vital for invasion in lung adenocarcinoma (55). It also has been shown that this protein has a negative prognostic role in pancreatic cancer (56). We suggest further studies on functional significance and mechanism of ACTA2 expression in esophageal cancer.

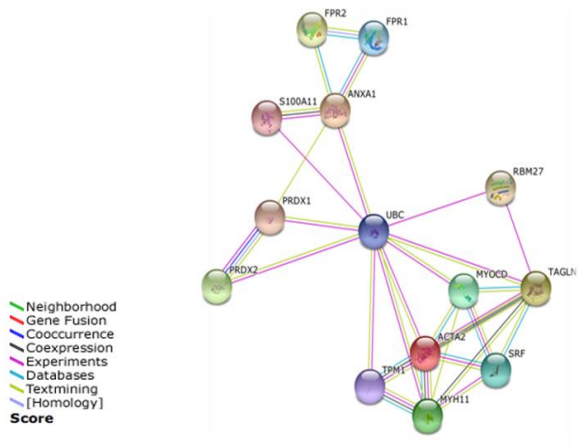

Figure 3. Protein–protein interaction maps of differentially expressed proteins identified in this study. Nodes are representative of protein species and different line colors show the types of evidence for the association. Interactive map of down-regulated and up-regulated proteins is listed in Table 1 plus ten related proteins. The STRING tool (http://www.string-db.org) was used to construct the interaction networks

Table 1. Differentially expressed proteins in EC tumor samples as identified by MALDI-TOF/TOF and MASCOT software

Protein Name	Accession No.	Mass	Score protein	Matches	Matched peptidsequence	Probability/ expectation value	Coverage	pI	Fold change tumor/normal	C.I%	Total Ion CI %	Total Ion score	Biological function (PANTHER classification)
Annexin A1GN=ANXA1	4502101	38918	125	5(4)	M.AMVSEFLK.Q	6.4e-09	22%	6.57	0.4	100	100	463	fatty acid metabolic process cell communication cytokinesis, mitosis, cellular component morphogenesis intracellular protein transport exocytosis, endocytosis cellular component organization
Actin, aortic smooth muscleGN=ACTA 2	4501883	42381	87	4(3)	K.AGFAGDDAPR.A	3.7e-05	15%	5.23	2.7	100	100	302	
Transgelin GN=TAGLN	119587704	22653	82	3(2)	.MANKGPSYGMSR.E	0.00012	19%	8.87	0.52	100	99.916	46	muscle contraction
Peroxiredoxin-2 GN=PRDX2	32189392	22049	86	5(5)	R.IGKPAPDFK.A	5.7e-05	23%	5.66	0.55	100	100	495	immune system process metabolic process

Conclusion

Four tumor-associated proteins that were identified in this study may provide useful information about the esophageal tumorigenesis. However, more studies with large populations of individuals with EC are needed to validate these potential biomarkers.

Conflict of interest

There is no conflict of interest about this article.

Acknowledgment

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