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World J Gastroenterol 2006 November 28; 12(44): 7104-7112
World Journal of Gastroenterology ISSN 1007-9327

ESOPHAGEAL CANCER

Identification of squamous cell carcinoma associated proteins by proteomics and loss of beta tropomyosin expression in esophageal cancer

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Received: 2006-08-02 Accepted: 2006-08-22

Abstract

AIM: To assess the proteome of normal versus tumor tissue in squamous cell carcinoma of the esophagus (SCCE) in Iranian patients and compare our results with former reports by using proteomics.

METHODS: Protein was extracted from normal and tumor tissues. Two dimensional electrophoresis was carried out and spots with differential expression were identified with mass spectrometry. RNA extraction and RT-PCR along with immunodetection were performed.

RESULTS: Fourteen proteins were found whose expression levels differed in tumor compared to normal tissues. Mass spectrometric analysis resulted in the identification of β-tropomyosin (TMβ), myosin light chain 2 (and its isoform), myosin regulatory light chain 2, peroxyredoxin 2, annexin I and an unknown polypeptide as the down regulated polypeptides in tumor tissue. Heat shock protein 70 (HSP70), TPM4-ALK fusion oncoprotein 2, myosin light polypeptide 6, keratin I , GH16431p and calreticulin were the up-regulated polypeptides found in tumor tissue. Several of these proteins, such as TMβ, HSP70, annexin I , calreticulin, TPM4-ALK and isoforms of myosins, have been well recognized in tumorigenesis of esophageal or other types of cancers.

CONCLUSION: Our study not only supports the involvement of some of the formerly reported proteins in SCCE but also introduces additional proteins found to be lost in SCCE, including TMβ.

Key words: Squamous Cell Carcinoma; Esophagus; Esophageal; Proteomics; Two dimensional electrophoresis; Polypeptide marker

Jazii FR, Najafi Z, Malekzadeh R, Conrads TP, Ziaee AA, Abnet C, Yazdznbod M, Karkhane AA, Salekdeh GH. Identification of squamous cell carcinoma associated proteins by proteomics and loss of beta tropomyosin expression in esophageal cancer. *World J Gastroenterol* 2006; 12(44): 7104-7112

http://www.wjgnet.com/1007-9327/12/7104.asp

INTRODUCTION

As a complex disease, cancer arises from a range of genetic alterations that result in both structural and functional cellular changes. Such alterations, including up and down regulation of certain genes, mutations, chromosomal alterations, and suppression or activation of specific genes, confer cells with growth advantage and clonal expansion[1,2]. As a result, cells may acquire the ability to produce new proteins and other proteins that normally are present may be modified, reduced, augmented or even eliminated[3,4]. Quantitative variations in protein abundance represent alteration in functional gene expression, which demands analysis. Proteomics has provided simultaneous analysis of a large number of cellular protein constituents and is the most powerful direct analytical method for protein detection and evaluation[5,6].

Squamous cell carcinoma of the esophagus (SCCE) is the sixth most common cancer in the world. However, in developing countries it ranks fourth and occurs with higher frequencies in certain regions of the world, such as Iran, China, South Africa and France. The last epidemiologic

report indicated the highest incidence rate of this cancer from Iran[8]. So far, many molecular studies have been carried out on this cancer and the role of oncogenes, tumor suppressors, chromosomal abnormalities and other molecular events have been investigated. Noteworthy among these molecular alterations is the down regulation of important tumor suppressor genes and consequently their protein products, such as P53, Retinoblastoma (RB), Mutated in Colon Carcinoma (MCC), and Deleted in Colon Carcinoma (DCC)[9], which further indicates the importance of Tumor Suppressors (TSs) in the carcinogenesis of this type of cancer.

The present report represents further extension of our earlier work on esophageal cancer[10]. Here we applied proteomics as a method of comprehensive analysis of differential protein abundances present in esophageal cancer. Among the fourteen differentially expressed proteins identified in this study, we observed loss of ß-tropomyosin (TMβ) in all tumor samples, a protein that has been suggested to function as a tumor suppressor. Furthermore, this study additionally supports the involvement of TSs in the etiology of SCCE.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma unless otherwise specified.

Patients and tissue sampling

Cancerous and normal tissue specimens were collected from 45 patients with SCCE whom underwent surgery. Tissue samples were collected immediately after surgery, wrapped in aluminum foil, snap frozen in liquid nitrogen and maintained at -70℃. The age of the patients at the time of diagnosis ranged from 27 to 86 years (63% males, 37% females) with a mean of 55 years.

Protein preparation

100-150 mg of tissue was sliced on ice and pulverized under liquid nitrogen using a microdismemberator (Braun, Germany). Subsequently 600 μL homogenization buffer (10 mmol/L Tris-HCL (BioRad), 5 mmol/L $MgCl_2$ PH 7.4) was added to the pulverized tissues, mixed and 10 μL of the following protease inhibitors were added: Pepestatin (1 mg/mL in isopropanol), benzamidine (16 mg/mL in H_2O), phenylmethylsulphonyl fluoride (PMSF at 25 mg/mL in isopropanol). To this homogenate, 10 μL of RNaseA (10 mg/mL in homogenization buffer) and DNase Ⅰ (1 mg/mL in homogenization buffer**)** were added and incubated on ice for 20 min. Subsequently, urea at 7 mol/L, thiourea at 2 mol/L, 5% ß-mercaptoethanol and 0.5% SDS were gradually added and the volume of solution was adjusted to 1.5 mL with the homogenization buffer. Samples were centrifuged at high speed for removal of insoluble particles and five μL of each was used for protein concentration assessment using the Bradford assay.

Two-dimensional electrophoresis

Samples were subjected to isoelectrofocusing (IEF) following to adaptations and slight modification[11]. The first dimension gel was composed of 4.2% acrylamide, 0.22% N, N'methylenebisacrylamide, 8.5 mol/L urea, 0.27% (V/V) Nonidet P-40 (NP-40), 5% sucrose, and 6% ampholytes (pH 4-6, 5-7 and 6-8 at a ratio of 2:1:2, respectively) and 0.05% TEMED (N, N, N', N'-Tetamethylethylene diamine). The solution was degassed and 0.04% ammonium persulfate was added, mixed and poured to a height of 130 mm in cylindrical glass tubes with a 1.5 mm internal diameter. A volume of sample equal to 75 μg total protein was mixed with 0.33 volume of neutralizing buffer (9 mol/L urea, 8% NP-40 and 5% ampholytes pH 3.5-10), loaded on IEF gel, overlaid with 10 μL sample buffer (4 mol/L urea, 1% ampholytes pH 3.5-10) and filled with catholyte. The upper chamber buffer or catholyte was composed of extensively degassed 0.02 mol/L NaOH and the lower chamber buffer or anolyte; 0.01 mol/L phosphoric acid. Isoelectric focusing was applied, without prefocusing, at 300 V for 1 h, afterward at 600 V for 10 h and 800 V for 1 h in order to final focusing. Gels were removed and equilibrated for 20 min at room temperature in equilibration solution (60 mmol/L Tris-HCl, pH 6.8, 2% SDS, 5% β- mercaptoethanol (V/V), 10% glycerol (V/V), and 0.002% bromophenol blue). The second dimension gel consisted of 33.3 mL of 30% stock acrylamide solution and N, N'-methylene bisacrylamide (29.2% and 0.8% W/V respectively), 41.7 mL deionized water and 25 mL separation gel buffer (1.5 mol/L Tris-HCl, pH 8.8, 0.4% SDS [Sodium Dodecyl Sulfate]), 0.034% (W/V) ammonium persulfate and 0.05% TEMED. The equilibrated first dimension gel was layered on the second dimension gel and fixed in place with 1% agarose and electrophoresis was carried out at 30 mA/plate at 10℃ constant temperature by applying a cooling system.

Protein detection

Proteins were detected using a slight modification of the previously reported method[11]. The gel was fixed (methanol, water, acetic acid and formaldehyde: 50/38/12/0.05 per volume) for at least 1h with constant shaking followed by 3 times 20 min washes with 50% ethanol, pretreated with sodium thiosulfate ($Na_2S_2O_3.5H_2O$; 0.2 g/L) for 1 min, and washed three times each for 20 s with ddH_2O. Impregnation of the gel with $AgNO_3$ (1.9 g/L and 0.075% (V/V) of 37% formaldehyde) was carried out and the residual $AgNO_3$ was removed by 3 × 20 s successive washes with de-ionized water. Successively the gel was developed by soaking in developing solution containing Na_2CO_3 (60 g/L), 0.05% (V/V) of 37% formaldehyde and 4 mg/L of $Na_2S_2O_3 \cdot 5H_2O$ for 10 min up to the appearance of yellowish brown spots. The gel was then rinsed twice, each for 2 min, with ddH_2O. Further development was stopped by immersing the gel in stop solution (50% methanol and 12% acetic acid) and stored in 30% ethanol at 4℃ until scanning.

Isoelectric point determination

The isoelectric point (pI) of polypeptides was determined by either application of protein PI markers (Sigma) or by determining PI change along the entire length of the first dimension gel[12]. Gels were cut into 0.5 cm long pieces. Each piece was placed in a separate tube containing 0.5

mL of distilled H_2O, crushed and incubated at room temperature for 2 h. The pH of each tube was then measured.

Image analysis

Silver-stained gels were scanned using a calibrated GS-800 densitometer (Bio-Rad) at resolution of 600 dots and 12-bits per inch. Spot detection and gel matching were done according to Melanie-4 default settings and spot pairs were investigated visually (GeneBio, Geneva, Switzerland). Pairs of Two dimensional electrophoresis (2DE) gels composed of tumor and normal gels from each patient were used and the percent volume of each spot was estimated and analyzed for image analysis. The polypeptide variants of each pair of gels (tumor versus normal) were recorded and the most common changes in the polypeptide expression pattern (70% or higher) in all 2DE gels were used as a reference for comparison between tumor and normal gels.

Immunological detection and localization of actin

Following to the 2DE, gels were equilibrated for 30 min in transfer buffer (25 mmol/L Tris-base, 192 mmol/L glycine, 20% methanol and 0.1% SDS) and polypeptides were electrophoretically transferred to nitrocellulose membranes at 14 V overnight. Membranes were blocked for 2 h in blocking solution (TBST; 100 mmol/L Tris-base or HCl, pH7.5, 0.9% NaCl and 0.05% Tween 20) and exposed to biotinylated anti-actin antibody at 1/3000 dilution for 1 h with constant shaking. Membranes were subsequently washed 3X with TBST, incubated with strptavidin conjugated alkaline phosphatase in TBST at 1/4000 dilution and further incubated (1 h) with constant shaking. Membranes were washed two times with TBST, once with TBS and exposed to color solution (33 μL NBT from 50 mg/mL stock solution in 70% dimethylformamide and 17 μL BCIP from 50 mg/mL stock solution in 100% dimethylformamide). To stop excess color formation, membranes were washed with distilled water[13].

RNA extraction and RT-PCR of TMβ and beta actin

Total RNA was extracted by acid phenol method and the first strand cDNA was synthesized applying oligo dT 18 (Roche). Subsequently amplification was carried out using the forward primer 5'-GGC TGA TGA GAG CGA GAG AG-3' and the reverse primer 5'-GCA CTG GCC AAG GTC TCT TC-3' for amplification of TMβ and 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3' as forward and 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3' as reverse primers for amplification of beta actin. The PCR condition was composed of primary denaturation at 95℃ 1 min followed by 30 cycles of amplification according to the subsequent scheme; denaturation 1 min at 95℃, annealing at 56℃ 1 min and extension at 72℃ 1 min and final extension at 72℃ for 10 min. Subsequently 4 μL of the PCR product was used for agarose gel electrophoresis.

Mass spectrometry

Silver stained protein spots containing the proteins of interest were destained thoroughly with 1% H_2O_2 (typically 1 min) and lyophilized to dryness[14]. Silver stain removal using H_2O_2 was performed to enhance peptide adsorption by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI)[14]. The dehydrated gel bands were hydrated with 15 μg/L (Promega, Madison, WI) of porcine trypsin in 25 mmol/L NH_4HCO_3, pH8.2 on ice for 45 min. Excess trypsin was removed; gel bands were covered with 25 mmol/L NH_4HCO_3, pH8.2 and incubated at 37℃ overnight. Tryptic peptides were extracted from the gel bands with 70% acetonitrile and 0.1% trifluoroacetic acid. Samples were desalted with C18 Zip Tips (Millipore, Bedford, MA) as per manufacturer's protocols. 0.5 μL of sample was co-crystallized with 0.5 μL of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 1% trifluoroacetic acid and spotted directly on a stainless steel MALDI target plate. Mass spectra were acquired using a MALDI-TOF/TOF mass spectrometer (Voyager 4700, Applied Biosystems, Foster City, CA). MALDI-TOF/TOF spectra were internally calibrated (< 20 ppm) using trypsin autolysis products. Post-acquisition baseline correction and smoothing was carried out using software provided with the TOF/TOF instrument. Spectra were submitted to Mascot (http://matrixscience.com) for peptide mass fingerprinting.

RESULTS

Protein extraction, 2DE and image analysis

Before doing 2DE, the concentrations of extracted proteins from tissue samples were determined. The protein extraction efficiencies were similar for tumor tissues and their adjacent normal nonmalignant tissues. The yields of extractions were approximately 80 μg/mg for both tumor and normal tissues. Subsequently seventy five micrograms of proteins from either tissue type were used in the first dimension. Because of the epithelial origin of squamous cell carcinoma, the mucosa of the normal non malignant tissues were separated from the rest of the esophageal tract for protein extraction and proteome comparison with corresponding tumors. For further verification that an equal amount of protein from either type of tissue was applied, immunological detection of actin was carried out following to 2DE. Following electrophoresis, the resolved proteins were recorded for each of the 2DE gel pairs. The densitometry map contained almost 800 features ranging in the molecular masses from 10 to 220 kDa and pIs from 4.8 to 8.0. For deciphering tumor-associated polypeptides, 2DE gels of tumor tissues were compared with their corresponding matched normal tissues. Compared with the matched normal tissues, 92 definite proteins (spots) in 2DE gels from 45 tumor tissues belonging to 45 patients were found indicating lower levels of expression. In contrast, 88 spots were identified in tumors that indicated higher levels of expression with respect to the 2DE gels of their corresponding matched normal tissues. To eliminate the possibility of artifacts, pairs of 2DE gels (tumor versus normal for each patient) were matched and compared and spot variants that occurred most often, or at least in 70% or higher of all 2DE patterns, were considered to be significant and were used for further comparisons. There were 7 out of 92 spots that indicated lower levels

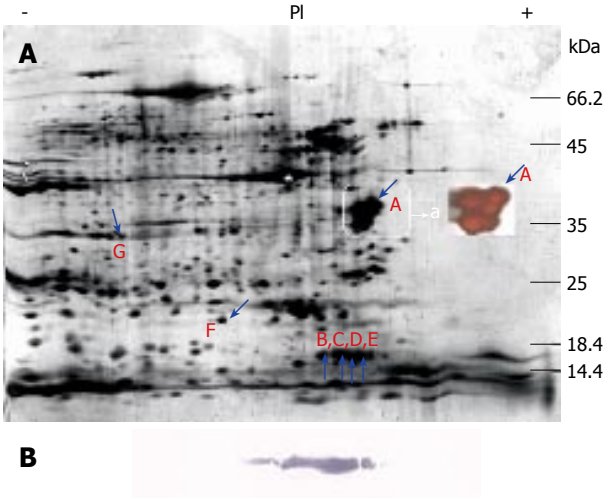

Figure 1 **A**: A representative 2DE gel of a normal tissue. Proteins that become down-regulated in corresponding tumor (Figure 2A) are shown with arrows and capital letters. For a better visualization of spots within the box, silver stained image of another gel is shown; **B**: Immunodection of actin as an internal control of protein loading, the * represents the location of actin.

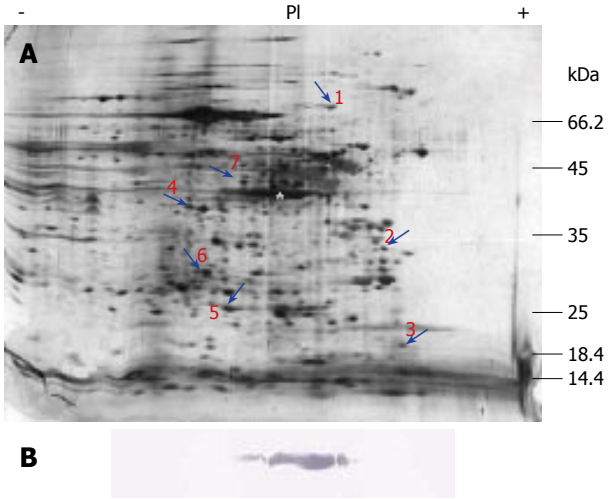

Figure 2 **A**: A representative 2DE gel of tumor tissue. Arrows and numbers indicate up-regulated proteins in comparison with their matched normal tissue (Figure 1A); **B**: Immunodetection of actin and the * represents the location of actin.

Table 1 Mass spectrometric identification and characteristics of the 14 proteins whose expression were subjected to change in SCCE

Polypeptide	Tissue specification	Protein name	Accession number based on NCBI	Chromosome	PI/MW	Expression profiling or level
A	Normal	Beta tropomyosin	NM-003289	9p13.2-p13.1	5.5/40.3	Disappearance
B	Normal	Myosin regulatory light chain 2	XM-027060	18p11.31	5.45/17.6	Down Regulation
C	Normal	Myosin light chain 2	NM-000432	16p11.2	5.6/17.6	Down Regulation
D	Normal	Unknown	NC-000963	Map No	5.8/17.6	Down Regulation
E	Normal	Myosin light chain 2	NM-000432	16p11.2	6/17.6	Down Regulation
F	Normal	Peroxiredoxin 2	NM-005809	19p13/2	6.6/26	Down Regulation
G	Normal	Annexin 1	NM-000700	9	7.3/35.5	Down Regulation
1	Tumor	Heat shock protein 70 kD	XM-044201	Unpublish	6/64	Up Regulation
2	Tumor	TPM4-ALK fusion oncoprotein 2	A186109	19p13/1	5.5/32.5	Up Regulation
3	Tumor	Myosin light polypeptide 6	NM-0211019	12p13/3	5.4/20.1	Up Regulation
4	Tumor	Keratin 1	AF304164	12q13	6.9/42.5	Up Regulation
5	Tumor	DNA directed RNA polymerase B (ropB)	NC-000915		6.1/27.2	Up Regulation
6	Tumor	GH16431P	Ay051511		6.6/30.1	Up Regulation
7	Tumor	Calreticulin	NM-004343	19p13/3	5.2/50	Up Regulation

of expression and 7 out of 88 spots that indicated higher levels of expression respectively. Figures 1A and 2A represent the resulting 2DE gels of the normal and the corresponding tumor tissues of one patient.

To identify whether an equal amount of protein was applied in 2DE, immunodetection of actin was carried out as an internal control of loading (Figures 1B and 2B). As Figures 1B and 2B show, equal amount of proteins were applied in the first dimension. The 14 observed differentially abundant proteins are indicated by arrows (Figures 1A and 2A). The proteins that were observed with decreased abundance in tumors, compared with their corresponding normal tissues, are labeled alphabetically as A, B, C, D, E, F and G. Spots B, C and E are observed in a chain of spots and likely indicate that they are isoforms or modified forms of a specific protein. On the other hand, those proteins that were observed to have an increased abundance in tumors were numbered as 1, 2, 3, 4, 5, 6 and 7. The apparent molecular weight of polypeptides, approximate isoelectric points and changes in expression level, is presented in Table 1. It was found that spot A was absent in tumor samples whereas, spots B, C, D and E were considerably down regulated and spots F and G were shown to be more than two-fold down regulated. It suggests that down regulated proteins were involved in the maintenance of normal phenotype whereas the other up-regulated polypeptides were involved in the development of malignancy.

Mass spectrometric analysis, identification of polypeptides and RT-PCR

Following to 2DE, spots of interest were excised, in-gel

Table 2 An example of MALDI/TOF/TOF mass spectrometry. The identified protein was β tropomyosin, one of the fourteen identified proteins as designated by letter A in Figure 1A

Calculated mass	Observed mass	Start Seq.	End Seq.	Sequence	Modification
846.4679	846.433	232	238	LKEAETR	
894.4679	894.4463	162	168	YEEVARK	
916.4734	916.4508	192	198	QLEEELR	
1107.5792	1107.5211	239	248	AEFAERSVAK	
1143.6116	1143.5856	190	198	ARQLEEELR	
1170.6727	1170.6464	169	178	LVILEGELER	
1243.6528	1243.6317	92	101	IQLVEEELDR	
1262.597	1262.5764	179	189	SEERAEVAESR	
1298.7677	1298.7423	168	178	KLVILEGELER	
1332.6389	1332.6127	78	90	ATDAEADVASLNR	
1343.6801	1343.6615	38	48	QLEEEQQALQK	
1399.7539	1399.7324	91	101	RIQLVEEELDR	
1443.8053	1443.7762	106	118	LATALQKLEEAEK	
1460.7339	1460.7119	77	90	KATDAEADVASLNR	
1471.775	1471.7477	38	49	QLEEEQQALQKK	
1488.74	1488.715	78	91	ATDAEADVASLNRR	
1493.7338	1493.7158	141	152	MELQEMQLKEAK	Oxidation (M)
1616.835	1616.8109	77	91	KATDAEADVASLNRR	
1671.8911	1671.8682	169	182	LVILEGELERSEER	
1702.8792	1702.8088	36	49	CKQLEEEQQALQKK	
1719.8582	1719.834	192	205	QLEEELRTMDQAL	Oxidation (M)
1727.8922	1727.8687	92	105	IQLVEEELDRAQER	
1799.9861	1799.9597	168	182	KLVILEGELERSEER	
1817.8776	1817.8594	153	167	HIAEDSDRKYEEVAR	
1883.9933	1883.9731	91	105	RIQLVEEELDRAQER	
1946.9963	1946.9734	190	205	ARQLEEELRTMDQALK	Oxidation (M)
2202.1248	2202.1091	106	125	LATALQKLEEAEKAADESER	
2414.252	2414.2375	169	189	LVILEGELERSEERAEVAESR	
2534.1636	2534.271	199	220	TMDQALKSLMASEEEYSTKEDK	
3751.876	3751.9631	252	284	TIDDLEETLASAKEENVEIHQTLDQTLLELNNL	

digested and analyzed with a MALDI/TOF/TOF (Matrix-assisted laser desorption/Ionization/ Time of Flight/ Time of Flight) mass spectrometer. The observed peptide mass spectra were analyzed for protein identification by peptide mass fingerprinting that resulted in the identification of the 14 proteins that are listed in Table 1. The identified protein corresponding to spot A was found to be β-tropomyosin as shown in Table 2. As mentioned above, this protein was found to be lost in tumors thus, for further validation of results achieved regarding with TMβ, RT-PCR was conducted by applying TMβ specific primers. As Figure 3A indicates, the 431 bp amplification product is only limited to the cDNA synthesized from RNA extracted from normal tissue. Applying beta actin as an internal control of RT-PCR, which gives rise to a 670 bp amplification product (Figure 3B), and running the extracted RNA (Figure 3C) indicate that lack of amplification in tumor tissues should be due to the loss of expression of TMβ. These observations further point out that down regulation of TMβ occurs at transcriptional level in SCCE. As discussed later in the Discussion section, TMβ is a down stream target for several oncogenes and functions as a tumor suppressor gene.

DISCUSSION

Former studies on SCCE have led to the identification of Annexin I [15,16], tumor rejection antigen (gp96)[16], clustrin[17], tropomyosin 3, retinoblastoma binding like protein and K506 binding protein[18] as potential markers of esophageal and other types of cancers, some of which were also identified in the present study for instance loss of TMβ in breast cancer[19], myosin light chain 2 isoforms in transformed osteosarcoma[20], peroxiredoxin Ⅱ in pancreatic adenocarcinoma[21], calreticulin in bladder[22] and colon cancers[23], HSP70 in hepatocellular[4] and keratin 1 in gastric carcinoma cell lines[24]. In addition to those proteins previously introduced for esophageal cancer, we identified new proteins, among which TMβ was found to be completely absent in all tumor samples. Therefore we suggest this protein as a potential marker for SCCE. Loss or down regulation of TMβ in cancer is not restricted to our study. Studies on other types of cancers such as oral tongue squamous carcinoma[25] and carcinomatous breast lesions[26] have also led to this observation.

Different studies have indicated that TMβ functions as a tumor suppressor. For example, reversion of neoplastic phenotype, anti-angiogenic activity[27,28], induction of slow growth rate, anchorage, cytoskeleton organization in breast cancer MCF7 cell lines[29] and anoikis[19] are all the consequence of TMβ expression. TMβ is required for cytoskeleton establishment and mediation of TGF-β mediated stress fiber formation. Alternatively, the Ras-ERK pathway antagonizes with TGF-β induced stress fiber formation by suppressing TMβ expression[30]. Indeed TMβ is a down stream target for the ras oncogene[31]. To the best of our knowledge, no strong evidence has been documented regarding with the involvement of the

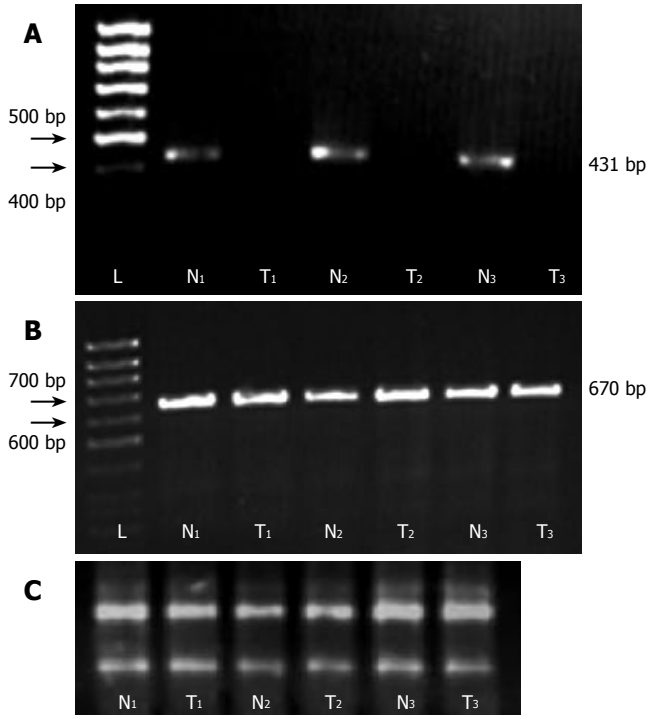

Figure 3 **A**: Verifying differential expression pattern of ß tropomyosin by RT-PCR in three separate experiments as indicated by numbers (1 to 3), normal versus tumor tissues. The amplification product (431bp) is limited to normal tissue which indicates loss or strong down regulation of this protein as observed by 2DE; **B**: RT-PCR amplification of ß-actin as an internal control of RT-PCR; **C**: Electrophoresis of the total RNA from normal and tumor tissues used for cDNA synthesis and RT-PCR. L: DNA marker; N: normal tissue; T: tumor tissue.

ras family of oncogenes in esophageal carcinogenesis, therefore it is likely that other oncogenes may be involved in the down regulation of TMβ, such as TGF-α and activation of EGFR, both of which participate in the down regulation of TMα and β[32]. Interestingly high levels of TGF-α were reported in all gastrointestinal cancers, including the late stages of esophageal cancer[33], along with amplification of EGFR in esophageal cancer[34]. Loss of TMβ in tumors suggests that this protein may play an important role in tumor suppression of esophageal carcinogenesis.

In addition to TMβ, down regulation of several other proteins was also observed in this study, including annexin Ⅰ. As we mentioned above, reports on the loss of annexins Ⅰ or its isoforms was not limited to our study but other research groups[15,16,37] have also observed this event. It was suggested that such a loss of expression in esophageal and prostate tumors is an early event[15] of carcinogenesis. Hypermethylation of annexin Ⅰ was suggested as another mechanism leading to inactivation and loss of expression in cell lines such as B cell non-Hodgkin's lymphoma[35]. Annexin Ⅰ induces both spontaneous and caspase-3 activated apoptosis[36]. Furthermore, mutations in p53 were also found to result to the down regulation of annexin 10, another member of the annexins family in hepatocellular carcinoma, along with vascular invasion, early recurrence and poor prognosis[38]. We formerly showed mutations in the p53 tumor suppressor gene in SCCE[10], thus it is possible that such mutations correlate with down regulation of annexin 10 as well as annexin Ⅰ. It is well-demonstrated that EGFR amplification occurs in SCCE[34]. On the other hand, annexin Ⅰ undergoes phosphorylation and inactivation by EGFR[39]. Thus our result additionally supports former report[16] regarding with loss of annexin Ⅰ in SCCE.

To our knowledge this is the first report introducing down regulation of peroxyredoxin Ⅱ in SCCE. Peroxiredoxins are members of the phase Ⅱ detoxifying enzymes involved in cytoprotection and cellular defense against oxidants by detoxifying xenobiotics and oxidants, especially H_2O_2. By reducing H_2O_2, peroxyredoxins protect cells from oxidative stress induced apoptosis and proapoptotic signals. Peroxyredoxins inhibit H_2O_2 induced signal cascade in response to PDGF and EGF. They also inhibit NFκB and tumor necrosis factor α in response to external H_2O_2 and H_2O_2 induced expression of proapoptotic protein; BAX[40,41]. Thus, it is possible that loss or down regulation of this protein or other members of detoxifying enzymes in esophageal epithelium, which normally could be exposed to stresses, contribute to the carcinogenesis by cellular failure in detoxification of oxidative agents affecting this tissue.

Loss of muscle forming proteins is an important cause of body weight loss or cachexia in cancers primarily due to loss of the myosin heavy chain[42]. Myosin light chain 2 is an indispensable component of cytoskeleton, migration and apoptosis[43]. Down regulation of myosin light chain 1 and myosin regulatory light chain 2 proteins was shown in oral tongue squamous cell carcinoma[25]. Due to a common embryonic origin of oral and esophagus tissues, strong down regulation of myosin regulatory chain 2 indicates an important role for this protein in both organs. Formerly Kumar and Chang have shown that transformation of osteosarcoma cells is associated with complete loss of myosin light chain 2 isoforms, which indicates myosin light chain 2 as a target for cellular transformation and malignancy[20]. Our report is the first demonstrating down regulation of myosin light chain isoforms in esophageal cancer. Alternatively, up regulation of myosin light polypeptide 6 in tumors could possibly be due to the selective down regulation of myosin heavy chain and other isoforms of myosin light chain. Supporting our results is the report of Samoszuk *et al* that have shown up regulation of this protein in clonogenic human breast cancer[44].

We also found several up regulated proteins in tumors, among which is heat shock protein 70. Investigations on gastric, hepatocellular and colonic adenocarcinoma cancers have indicated higher levels of expression of HSP70 along with other stress associated proteins[4,45]. HSP70 exerts antiapoptotic activity by suppressing tumor necrosis factor induced apoptosis and caspase 3 down stream events[46], interfering with SAPK/JNK and ceramide induced apoptosis and cleavage of caspase 3 substrate enzyme PARP, which is involved in DNA repair[47]. Theses findings have raised the idea that HSP70 not only should be considered as a tumor marker for HCV-caused hepatocellular carcinoma[48] but also as a target for cancer treatment[49]. Thus, as an organ of the digestive system, esophagus carcinogenesis might follow the same molecular path as other organs of this system.

Calreticulin and keratin 1 were among other up

regulated proteins in tumors. The involvement of calreticulin in tumors has been reported by several groups. Rendon Huerta *et al*[50] have shown that protein kinase C isoforms bind and phosphorylate calreticulin. This would suggest a possible role for calreticulin in signal transduction and involvement in cell division. It was also found that calreticulin regulates p53 function by affecting its rate of degradation and nuclear localization[51]. Thus, association of p53 mutations with esophageal carcinogenesis[10] as well as p53 degradation could explain the role of calreticulin in the development of esophageal tumor. In addition to calreticulin we also found overexpression of Keratin 1, a member of keratin family in SCCE. Formerly Trask and colleagues have shown different expression patterns of keratin isoforms in normal versus tumor tissues[52]. They proposed the expression pattern of keratin isoforms as biomarkers for differentiating normal from malignant cells. Furthermore, Kinjo and coworkers have shown overexpression of keratin 1 in colonic adenocarcinoma cells[53] and Nishikawa *et al*[24] have found up to 17 folds of truncated keratin 1 in Epstein-Barr virus transfected gastric carcinoma cell lines. In esophageal cancer, several groups have reported an increased level of cytokeratin 19 (keratin 19) in serum and secretion by cell lines[54]. The later groups have proposed cytokeratin 19 to have the best specificity and sensitivity as a prognostic marker for evaluating esophageal cancer. Thus involvement of another member of the keratin family in SCCE not only supports former results but also introduces a new marker from this family.

Activation of anaplastic leukemia kinase (ALK) is an example of chromosomal abnormalities, such as translocations, that lead to fusion proteins. ALK was first identified in anaplastic large cell lymphomas (ALCL) and subsequently in inflammatory myofibroblastic tumors (ITM). The aberrantly expressed protein activates several protein kinases, such as IP3 kinase, STAT5 and transcription factors, which end up to mitogenic effects[55]. The fusion of ALK with nucleoplasmin is frequently observed in ALCL. Walking on chromosome 2, Lamant *et al* have shown the fusion of tropomyosin 3 and ALK as a result of t (1; 2) (q25; p23)[56]. They suggested that such a fusion provides an active promoter for ALK, because nucleoplasmin is a housekeeping gene and TM3 is constitutively expressed. Further studies led to the identification of not only TM3-ALK but also TM4-AKL fusion proteins in patients with inflammatory myofibroblastic tumors[57] and as a result perturbation of normal tumor suppressor activity of TM isoforms. It was also suggested that the extent of such rearrangements and fusions could possibly engage other proteins. Here we demonstrated that expression of ALK is not restricted only to the neural but also to other tumors. TPM4-ALK fusion protein is also among proteins being introduced for the first time in SCCE.

There were also three other proteins found in SCCE as homologues of formerly identified proteins in other organisms. Thus, we interpret them to be newly found proteins in SCCE. Among these proteins, two were up regulated in tumors that were identified to be DNA-directed RNA polymerase Beta subunit (ropβ) with almost 100% (99.998) homology with that of *H pylori*. The other protein, spot 6 had a mass spectrum that matched with a high score (99.925) with GH16431P of Drosophila melanogaster (NCBI ACCESSION; AAK92935). Looking into patients pathology files indicated that almost all were *H Pylori* positive, thus we think that up regulation of this protein in tumors, to some extent, may be due to contamination with this bacterium. However, the question that remains unanswered is why such contamination was not observed in normal tissues. A possible explanation could be a preferentially higher growth of bacteria in tumors than in normal tissues. The other protein was the spot D in normal tissue whose mass spectrum matched with high score (99.963) to an unknown protein (NCBI Accession; NC 000963). This protein is highly expressed in normal tissue while loss of it was observed in tumor specimens. Being an unknown protein, further studies are required to reveal its true biological activity in tumor suppression.

In conclusion, this is the first report on the proteomics of SCCE from Iran, a country with the highest incidence rate of SCCE[8]. We were able to identify fourteen differentially expressed proteins in esophageal tumors. Our study further showed that several proteins, which are commonly affected in other digestive organs such as liver, are also affected in SCCE, which may indicate a common molecular mechanism for digestive system tumorigenesis and putative candidate biomarkers. It should also be mentioned that our study brings further evidence for the importance and requirement of a deeper investigation of the molecular events down stream to the EGFR activation in SCCE. The expression levels of several proteins identified in our study is affected by the activation of this receptor. To our knowledge a general molecular marker has not been introduced for cancers, however, finding the disappearance of TMβ in SCCE could introduce a useful tumor marker for SCCE diagnosis, evaluation and follow up.

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S- Editor Wang GP **L- Editor** Lutze M **E- Editor** Liu WF