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Identification of Squamous Cell Carcinoma Associated Proteins by Proteomics and Loss of Beta Tropomyosin Expression in Esophageal Cancer

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Running title: Proteomics of squamous cell carcinoma of esophagus

Abstract: *AIM: To assess the proteome of normal versus tumor of squamous cell carcinoma of esophagus (SCCE) in Iran and evaluate our results with other former reports by using proteomics.*
METHODS: Protein was extracted from normal and tumor tissues. Two dimensional electrophoresis (2DE) was carried out and spots with differential expression were identified with mass spectrometry. RNA extraction and RT-PCR along with immunodection were carried out for evaluating results.
RESULTS: Fourteen proteins were found whose expression levels differed in tumor compared with normal tissues. Mass spectrometric analysis resulted to the identification of ß-tropomyosin (TMß), myosin light chain 2 (and its isoform), myosin regulatory light chain 2, peroxyredoxin 2, annexin I and as yet an unknown polypeptide as down regulated polypeptides in tumors, whereas Heat shock protein 70 (HSP70), TPM4-ALK fusion oncoprotein 2, myosin light polypeptide 6, keratin I, GH16431p and calreticulin were the up-regulated polypeptides in tumors. The role of several of these proteins such as TMß, HSP70, annexin I, calreticulin, TPM4-ALK and isoforms of myosins has well been 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 for the first time among which TMß was found to be lost in SCCE.

Keywords*: Squamous Cell Carcinoma; Esophagus; Esophageal; Proteomics; Two dimensional electrophoresis; Polypeptide marker*

1. Introduction

As a complex disease, cancer arises from a range of genetic alterations resulting to changes both in structure and function of the cell. Such changes include up and down regulation of certain genes, mutations, chromosomal alterations, suppression or activation of specific genes, that overall confer cells with growth advantage and clonal expansion [1, 2]. As a result, cells acquire the ability to produce new proteins, but others which normally are present may be modified, reduced, augmented or even eliminated [3, 4]. Proteomics has provided simultaneous analysis of a large number of cellular protein constituents as well as the most powerful direct analytical method of protein detection and evaluation (5, 6).
However in developing countries, it ranks fourth and occurs with high frequencies in certain regions of the world, such as Iran, China, South Africa and France characterized with five-year survival rate as low as 8% (7,8). Noteworthy among these molecular alterations is the down regulation of the important tumor suppressor genes and consequently their protein products such as protein 53KDa (P53), Retinoblastoma(RB), Mutated in Colon Carcinoma (MCC), and Deleted in Colon Carcinoma(DCC) [9] which further indicate the importance of Tumor Suppressor (TSs) in the carcinogenesis of this type of cancer. The present report represents an extension of our earlier work on esophageal cancer (10) .

2. Materials And Methods

2.1. Chemicals

All chemicals were purchased from Sigma unless otherwise specified.

2.2. Patients and tissue sampling

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 mean of 55 years.

2.3. Protein preparation

100-150 mg of tissues were 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 MgCl2 PH 7.4) was added to the pulverized tissues, mixed and 10 µL of the following protease inhibitors: pepestatin (1 mg/mL in isopropanol), benzamidine (16 mg/mL in H2O), phenylmethylsulphonyl fluoride (PMSF at 25 mg/mL in isopropanol) were added. 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 microliters of which was used for protein concentration assessment using Bradford assay.

2.4. Two-dimensional electrophoresis

Samples were subjected to isoelectrofocusing (IEF) following to adaptation and slight modifications[11].

2.5. Protein detection

Proteins were detected using a slight modification of the previously reported method [11].

2.6. Isoelectric point determination

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 first dimension gel[12].

2.7. Image analysis

Silver-stained gels were scanned using 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).

2.8. Immunological detection and localization of actin

Following to 2-DE, gels were equilibrated for 30 min in transfer buffer and polypeptides were electrophoretically transferred to the nitrocellulose membranes at 14 V overnight. Membranes were blocked for 2 h in blocking solution 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. To stop excess color formation membranes were washed with distilled water([13].

2.9. RNA extraction and RT-PCR of TMß and beta actin

Total RNA was extracted by acid phenol method and the first strand cDNA was synthesized applying oligo d $(T)_{18}$ (Roche). Subsequently amplification was carried out using 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 TMß 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. PCR condition was composed of primary denaturation at 95℃ 1 min followed by 30 cycles of amplification according to subsequent scheme; denatuartion 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.

2.10. 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). Mass spectra were acquired using a MALDI-TOF/TOF mass spectrometer (Voyager 4700, Applied Biosystems, Foster City, CA). Spectra were submitted to Mascot (http://matrixscience.com) for peptide mass fingerprinting.

3. Results

3.1. Protein extraction, 2DE and image analysis

For further verification whether an equal amount of protein from either types of tissues were applied, immunological detection of actin (Figurers 1B and 2B) was carried out following to 2DE as internal control of loading. 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. Comparing with the matched normal tissues, 92 definite proteins (spots) in 2DE gels from 45 tumor tissues belonging to 45 patients were found becoming subjected to lower levels of expression. These spots were 7 out of 92 spots that have undergone lower levels of expression and 7 out of 88 spots which have shown higher levels of expression respectively. Figures 1A and 2A represent the resulting 2DE gels of the normal and the corresponding tumor tissues of one of the patients. 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 form 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 and spots F and G were shown more than two folds of down regulation. It is suggested that down regulated proteins to be involved in the maintenance of normal phenotype whereas the other up-regulated polypeptides in development of malignanancy.

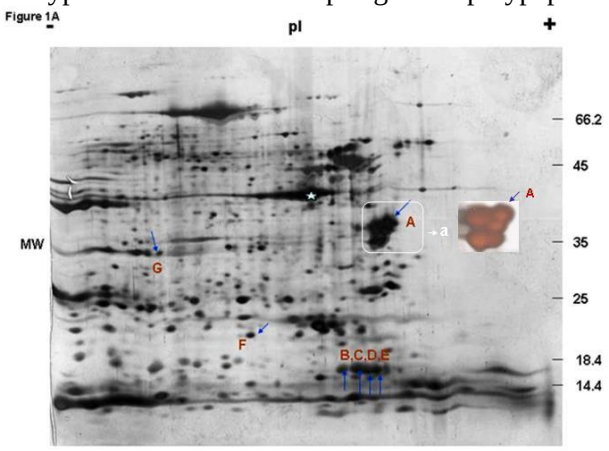


Fig. 1A :

Fig. 1B

Fig. 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.

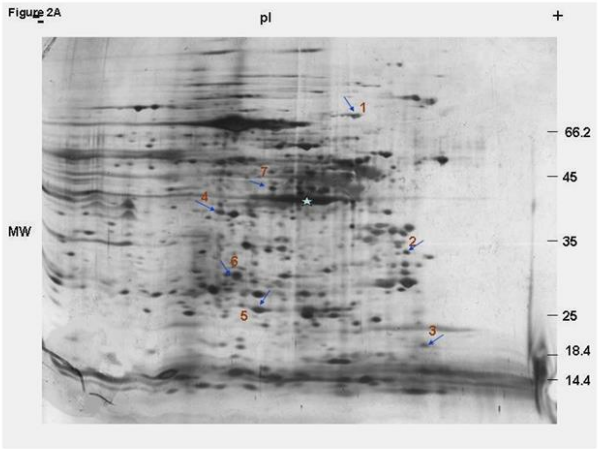


Fig. 2B

Fig. 2B:

Fig. 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.

3.2. Mass spectrometric analysis, identification of polypeptides and RT-PCR

Following to 2DE, spots of interest were excised, in gel digested and analyzed with MALDI/TOF/TOF(Matrix-assistedlaser 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 to the identification of all the 14 proteins which are listed in Table 1. The identified protein corresponding to spot A was found to be ß-tropomyosin . As was mentioned above this protein was found to be lost in tumors thus for further validating results achieved regarding with TMß, RT-PCR was conducted by applying TMß specific primers. As Figure 4A indicates the 431bp 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 670bp amplification product (Figure 4B) along with running the extracted RNA (Figure 4C) indicate that lack of amplification in tumor tissues should be due to loss of expression of TMß. These observation further points out that down regulation of TMß occur at transcriptional level in SCCE. As discussed later on in discussion, TMß is a down stream target for several oncogenes and function as a tumor suppressor gene.

Fig. 3:

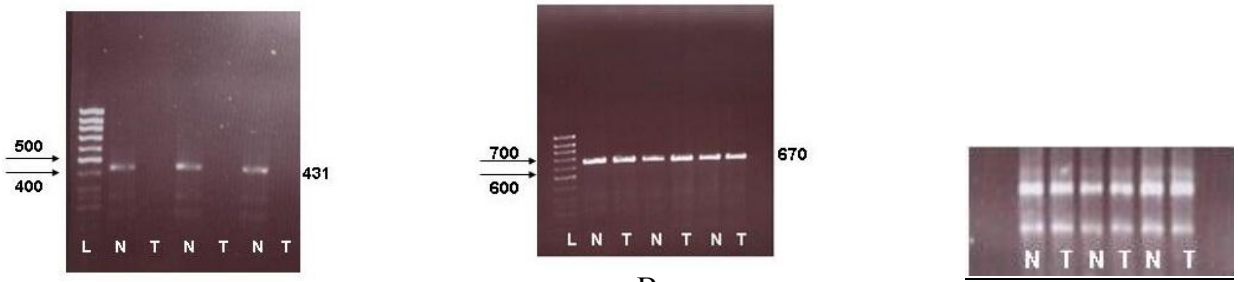
Rank	Protein Name	Accession No.	Protein Score	Protein C. I. %
1	(NM_003289) tropomyosin 2 (beta) [Homo sapiens]	gi|4507649	338	100

Peptide Information

Calc. Mass	Obsrv. Mass	Start Seq.	End Seq.	Sequence	Modification
	916.4734	916.4508	192	198	QLEEELR
	#				
	1471.775	1471.7477	38	49	QLEEEQQALQKK
	1488.74	1488.715	78	91	ATDAEADVASLNRR
	1493.7338	1493.7158	141	152	MELQEMQLKEAK 1 Oxidation (M)
	1719.8582	1719.834	192	205	QLEEELRTMDQALK 1 Oxidation (M)
	1727.8922	1727.8687	92	105	IQLVEEELDRAQER
	1799.9861	1799.9597	168	182	KLVILEGELERSEER
		of	**92**		
	1883.9933	1883.9731	91	105	RIQLVEEELDRAQER
	1946.9963	1946.9734	190	205	ARQLEEELRTMDQALK 1 Oxidation (M)
	2202.1248	2202.1091	106	125	LATALQKLEEAEKAADESER

Fig. 3 An example of MALDI/TOF/TOF mass spectrometry. The identified protein was ß tropomyosin, one of the fourteen identified proteins as designated by letter A in Figure 1A.

Fig. 4



A B C

Fig. 4 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.

4. 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] For instance loss of TMß 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]. 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 TMß 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 and anoikis[29] are all the consequence of TMß expression. TMß is required for cytoskeleton establishment andmediation of TGF-ß mediated stress fiber formation. Alternatively Ras-ERK pathway antagonizes with TGF-ß induced stress fiber formation by suppressing TMß expression [30]. Indeed TMß is a down stream target for ras oncogene [31]. To the best of our knowledge, no strong evidence has been documented regarding with the involvement of the ras family of oncogenes in esophageal carcinogenesis, therefore it is likely that other oncogenes may be involved in down regulation of TMß such as TGF-a and activation of EGFR; both of which participate in down regulation of TMa and ß [32]. Interestingly high level of TGF-a was reported in all gastrointestinal cancers including the late stages of esophageal cancer [33] along with amplification of EGFR in esophageal cancer [34]. Loss of TMß in tumors suggests that this protein may play an important role in tumor suppression of esophageal carcinogenesis.

In addition to TMß, down regulation of several other proteins was also observed in this study, among which annexin I. Loss of annexins I expression was reported in tumors including esophagus and prostate as an early event [15] and in cell lines such as B cell non Hodgkin's lymphoma along with hypermethylation of promoter [35]. Annexin I induces both spontaneous and caspase 3 activated apoptosis [36]. Zhi *et al* [37] have demonstrated inhibition of growth, colony formation and tumorigenesis of EC9706; an esophageal carcinoma cell line in nude mice following to transfection with annexin I. Furthermore mutations in p53 were also found to result in down regulation of annexin 10, another member of 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 undergo phosphorylation and inactivation by EGFR [39]. Thus our result additionally supports former report [16] regarding with loss of annexin I in SCCE.

They also inhibit NF?B and tumor necrosis factor a 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 important cause of body weight loss or cachexia in cancers primarily due to loss of myosin heavy chain [(42)]. Myosin light chain 2 is 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)]. Supporting our results is the report of Samoszuk *et al.* that have formerly shown up-regulation of this protein in clonogenic human breast cancer [(44)].

We also found several up-regulated proteins in tumors, among which heat shock protein 70. Investigations in cancers such as gastric, hepatocellular and colonic adenocarcinoma 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 has 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)].

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. Added to calreticulin we found overexpression of Keratin 1, a member of keratin family in SCCE. Formerly Trask and colleagues have shown different expression pattern of keratin isoforms in normal versus tumors[(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 keratin family in SCCE not only supports former results but also introduces a new marker from this family.

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 chromosme 2, Lamant *et al.* have shown the fusion of tropomyosin 3 and ALK as a result of t(1;2) (q25;p23) [(56)]. 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

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 SCCEIn conclusion this is the first report on proteomic analysis 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 as well as liver are also affected in SCCE which may additionally explain a common molecular mechanism for digestive system tumorigenesis and putative candidate biomarkers. To our knowledge while up to now not a general molecular marker has been introduced for cancers, however finding disappearance of TMß in SCCE could introduce a useful tumor marker for SCCE diagnosis, evaluation and follow up.

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6. References

[1] Gray JW, Collins C. Genome changes and gene expression in human solid tumors. *Carcinogenesis* 2000; 3: 443 –452.[PMID: 10688864]

http://dx.doi.org/10.1093/carcin/21.3.443
[2] Tahara E. Encyclopedia of Molecular Cell Biology and Molecular Medicine. Growth factors and oncogenes in gastrointestinal cancers. 2nd Ed. Volume 6. Edited by Robert. A. Mayers, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. 2005.p 1-32.
[3] Hondermarck H, Sophie A, Edouart V, Revillion F, Lemoine J, Ei-Yazidi-Belkoura I. Proteomics of breast cancer for marker discovery and signal pathway profiling. *Proteomics* 2001; 1:1216-232.[PMID: 11721634]
http://dx.doi.org/10.1002/1615-9861(200110)1:10<1216::AID-PROT1216>3.0.CO;2-P
http://dx.doi.org/10.1002/1615-9861(200110)1:10<1216::AID-PROT1216>3.3.CO;2-G
[4] Kim W, Lim SO, Kim JS, Ryu YH. Comparison of proteome between hepatitis B virus and hepatitis C virus associated hepatocellular carcinoma.. *Clinical Cancer Research* 2003 ;9:5493-5500.[PMID: 14654528]
[5] Anderson NG, Matheson A, and Leigh-Anderson N. Back to the future: The human protein index(HPI) and the agenda for post proteomics biology. *Proteomics* 2001;1:3–12.[PMID: 11680895]
http://dx.doi.org/10.1002/1615-9861(200101)1:1<3::AID-PROT3>3.3.CO;2-K
http://dx.doi.org/10.1002/1615-9861(200101)1:1<3::AID-PROT3>3.0.CO;2-T
[6] Srinivas PR, Verma M, Zhao Y, and Srivastava S. Proteomics for cancer biomarker discovery. *Clinical Chemistry* 2002;8:1160 –1169.[PMID: 12142368]
[7] Blot WJ. Esophageal cancer, trends and risk factors. *Seminars in Oncology* 1994;21:403–410.[PMID: 8042037]
[8] Munoz N. Epidemiological aspects of oesophageal cancer. *Endoscopy* 1993;25:609–612.[PMID: 8119215]
http://dx.doi.org/10.1055/s-2007-1010415
[9] Rosen N. The molecular basis of cellular transformation: implications for esophageal. *carcinogenesis* 1994;21 :416-424.[PMID: 8042039]
[10] Sepehr A, Taniere P, Martel-Planche G, Ziaee AA, Rastgar-Jazii F, Yazdanbod M,. Distinct pattern of TP53 mutations in squamous cell carcinoma of the esophagus in Iran. *Oncogene* 2001;20:7368-7374.[PMID: 11704866]
http://dx.doi.org/10.1038/sj.onc.1204912
[11] Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 1987;8:93-99.
http://dx.doi.org/10.1002/elps.1150080203
[12] Andrews AT. Electrophoresis, theory, techniques and biochemical and clinical applications, 1990. P 267.
[13] John E, Coligan Ada M, Kruisbeek David H, Margulies Ethan M, Shevach Warren Strober. *Current Protocols in Immunology*. New York: John Wiley and Sons;1994. p: 8.10.1-8.10.6.
[14] Sumner LW, Wolf-Sumner B, White SP,and Asirvatham VS. Silver stain removal using H2O2 for enhanced peptide mass mapping by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Communication in Mass Spectrometery* 2002:16:160-168.[PMID: 11803536]
http://dx.doi.org/10.1002/rcm.555
[15] Paweletz CP, Ornstein DK, Roth MJ, Bichsel VE, Gillespie JW, Calvert VS, Vocke CD, Hewitt SM, Duray PH, Herring J, Wang QH, Hu N, Linehan WM, Taylor PR, Liotta LA, Emmert-Buck MR, Petricoin EF 3rd. Loss of annexin I correlates with early onset of tumorigenesis in esophageal and prostate cancer. *Cancer Research* 2000;60:6293-6297.[PMID: 11103786]
[16] Zhou G, Li H, DeCamp D, Chen S, Shu H, Gong Y, Flaig M, Gillespie JW, Hu N, Taylor PR, Emmert-Buck MR, Liotta LA, Petricoin EF 3rd, Zhao Y. 2D differential in gel electrophoresis for the identification of esophageal scans cell cancer specific protein markers. *Molecular and Cellular Proteomics* 2002; 1:117-124. [PMID: 12096129]
http://dx.doi.org/10.1074/mcp.M100015-MCP200
[17] Zhang LY, Ying WT, Mao YS, He HZ, Lio Y, Wang HX. Loss of clustrin both in serum and tissue correlates with the tumorigenesis of esophageal squamous cell carcinoma via proteomics approaches. *World Journal of Gastroenterology* 2003;4:650-654.[PMID: 12679903]
http://dx.doi.org/10.3748/wjg.v9.i4.650

[18] Xiong XD, Li EM, Xu LY, Chen HB, Chen L, Cai WJ. Separation and identification of differentially expressed nuclear matrix proteins between human esophageal immortalized and carcinomatous cell lines. *World Journal of Gastroenterology* 2003;10:2143-2148.[PMID: 14562366]
http://dx.doi.org/10.3748/wjg.v9.i10.2143

[19] Raval GN, Bharadwaj S, Levine EA, Willingham MC, Geary RL, Kute T. Loss of expression of tropomyosin 1, a novel class II tumor suppressor that induces anoikis, in primary breast tumors.*Oncogene* 2003;22:6194-6203.[PMID: 13679858]
http://dx.doi.org/10.1038/sj.onc.1206719

[20] Kumar CC, and Chang C. Human smooth muscle myosin light chain-2 gene expression is repressed in *ras* transformed fibroblast cells. *Cell Growth and Differentiation* 1992;3:1-10.[PMID: 1599878]

[21] Shen J, Person MD, Zhu J, Abbruzzese JL, and Li D. Protein expression profiles in pancreatic adenocarcinoma compared with normal pancreatic tissue and tissue affecter by pancreatitis as detected by two dimensional gel electrophoresis and mass spectrometry. *Cancer Research* 2004;64:9018-9026.[PMID: 15604267]
http://dx.doi.org/10.1158/0008-5472.CAN-04-3262

[22] Kageyama S, Isono T, Wakabayashi Y, Okada Y, Yoshimura K, Terai A, Arai Y, Iwamura H, Kawakita M, Yoshiki T. Identification by proteomic analysis of calreticulin as a marker for bladder cancer and evaluation of the diagnostic accuracy of its detection in urine. *Clinical Chemistry* 2004;50:5:857-866.[PMID: 14764641]
http://dx.doi.org/10.1373/clinchem.2003.027425

[23] Brunagel C, Shah U, Schonen RE, and Getzenberg R. Identification of calreticulin as a nuclear matrix protein associated with human colon cancer .*Journal of Cellular Biochemistry* 2003;89:238-243.[PMID: 12704787]
http://dx.doi.org/10.1002/jcb.10502

[24] Nishikawa J, Kiss C, Imai S, Takada K, Okita K, Klein G. Upregulation of the truncated basic hair keratin 1(hHb1-ΔN) in carcinoma cells by Epstein Barr virus (EBV). *International Journal of Cancer* 2003;107:597-602.[PMID: 14520698]
http://dx.doi.org/10.1002/ijc.11289

[25] He QY, Chen J, Kung HF, Yuen AP, Chiu JF. Identification of tumor associated proteins in oral tongue squamous cell carcinoma by proteomics. *Proteomics* 2004;1:271–278.[PMID: 14730689]
http://dx.doi.org/10.1002/pmic.200300550

[26] Franzen B, Linder S, Uryu K, Alaiya AA, Hirano T, Kato H. Expression of tropomyosin isoforms in benign and malignant human breast lesions. *British Journal of Cancer* 1996;73:909-913.[PMID: 8611405]
http://dx.doi.org/10.1038/bjc.1996.162

[27] Prasad GL, Fuldner RA, Cooper HL. Expression of transduced tropomyosin 1 cDNA suppresses neoplastic growth of cells transformed by the *ras* oncogene. *Proceedings of the National Academy of Sciences U.S.A* 1993 ;90:7039-7043.[PMID: 8346214]
http://dx.doi.org/10.1073/pnas.90.15.7039

[28] Shah V, Bharadwaj S, Kaibuchi K, and Prasad GL. Cytoskeletal organization in tropomyosin mediated reversion of *ras* transformation: evidence for Rho kinase pathway.*Oncogene* 2001;20:2112-2121.[PMID: 11360195]
http://dx.doi.org/10.1038/sj.onc.1204291

[29] Mahadave K, Raval G, Bharadwaj S, Willingham MC, Lange EM, Vonderhaar B, Salomon D, Prasad GL. Suppression of the transformed phenotype of breast cancer by tropomyosin 1. *Experimental Cell Research* 2002;279:40-51.[PMID: 12213212]
http://dx.doi.org/10.1006/excr.2002.5583

[30] Bakin AV, Safina A, Rinehart C, Daroqui C, Darbary H, and Helfman DM. A critical role of tropomyosins in TGF-β regulation of the actin cytoskeleton and cell motility in epithelial cells. *Molecular Biology of the Cell* 2004;15:4682-4694.[PMID: 15317845]
http://dx.doi.org/10.1091/mbc.E04-04-0353

[31] Janssen RA, Kim PN, Mier JW, and Morrison DK. Overexpression of kinase suppressor of Ras upregulates the high molecular weight tropomyosin isoforms in *ras* transformed NIH3T3 fibroblasts. *Molecular and Cellular Biology* 2003;23:1786-1797.[PMID: 12588996]
http://dx.doi.org/10.1128/MCB.23.5.1786-1797.2003

[32] Shields JM, Mehta H, Pruitt K, and Der CJ. Opposing roles of the extracellular signal regulated kinase and p38- mediated activated protein kinase cascades in Ras mediated downregulation of tropomyosin. *Molecular and Cellular Biology* 2002;7:2304-2317.[PMID: 11884615]
http://dx.doi.org/10.1128/MCB.22.7.2304-2317.2002

[33] Moskal TL, Huang S, Ellis LM, Fritsche JR, and Chakrabarty S. Serum levels of transforming growth factor alpha in gastrointestinal cancer patients. *Cancer Epidemiology Biomarkers and Prevention*. 1995;4:127-131.[PMID: 7742720]

[34] Stoner GD, and Gupta A. Etiology and chemoprevention of esophageal squamous cell carcinoma. *Carcinogenesis* 2001;.11:1737-1746.[PMID: 11698334]

[35] Vishwanatha J, Salazar E, and Gopalakrishnan VK. Absence of annexin I expression in B-cell non-Hodgkin's lymphomas and cell lines. *BioMed Cancer* 2004;4:1-8.[PMID: 15070421]

[36] Solito E, Coupade Cd, Canaider S, Goulding NJ, Perretti M. Transfection of annexin 1 in monocytic cells produce a high degree of spontaneous and stimulated apoptosis associated with caspase-3 activation. *British Journal of Phrmacology* 2001;133:217-228.[PMID: 11350857]
http://dx.doi.org/10.1038/sj.bjp.0704054

[37] Zhi H, Ding F, Wang M, Wu M, and Liu Z. Suppression of Growth and Tumorigenicity in Human Esophageal Carcinoma Cell Line EC9706 by Overexpression of Annexin A1. *Annexins* 2004;1:121-128.

[38] Liu SH, Lin CY, Peng SY, Jeng YM, Pan HW, et al. Down regulation of annexin A10 in hepatocellular carcinoma is associated with vascular invasion, early recurrence and poor prognosis in synergy with p53 mutation. *American journal of pathology* 2002;5:1831-1837.[PMID: 12000734]
http://dx.doi.org/10.1016/S0002-9440(10)61129-7

[39] Pepinsky RB, and Sinclair K. Epidermal growth factor dependent phosphorylation of lipocortin. *Nature*. 1986;321:81-84.[PMID: 3010133]
http://dx.doi.org/10.1038/321081a0

[40] Kang SW, Chae HZ, Seo MS, Kim K, Baines IC, and Rhee SG. Mammalian peroxirdoxin isoforms can reduce hydrogen peroxide generated in respons to growth factors and tumor necrosis factor- α. *Journal of Biological Chemistry* 1998;273:6297-6302.[PMID: 9497357]
http://dx.doi.org/10.1074/jbc.273.11.6297

[41] Kim H, Lee TH, Park ES, Suh JM, Park SJ. Role of peroxredoxins in regulating intracellular hydrogen peroxide and hydrogen peroxide induced apoptosis in thyroid cells. *Journal of Biological Chemistry* 2000;275: 18266-18670. [PMID: 10849441]
http://dx.doi.org/10.1074/jbc.275.24.18266

[42] Acharyya S, Ladner KJ, Nelsen LL, Damrauer J, Reiser PJ, Swoap S. Cancer cachexia is regulated by selective targeting of skeletal muscle gene product. *The Journal of Clinical Investigation* 2004;114:370–378.[PMID: 15286803]
http://dx.doi.org/10.1172/JCI200420174

[43] Kaneko K, Satoh K, Masamune A, Satoh A, and Shimosegawa T. Myosin light chsin kinase inhibitors can block invasion and adhesion of human pancreatic cancer cell lines. *Pancreas* 2002;1:41-43.[PMID: 11741180]

[44] Samoszuk M, Tan J, Chorn G. Clonogenic growth of human breast cancer cells co-cultured in direct contact with serum activated fibroblasts. *Breast Cancer Research* 2005;7:74-83.[PMID: 15987422]
http://dx.doi.org/10.1186/bcr995

[45] Wang XP, Qiu FR, Lio GZ, Chen RF. Correlation between clinicopathology and expression of heat shock protein 70 and glucose regulated protein 94 in human colonic adenocarcinoma. *World Journal of Gastroenterology* 2005;11:1056–1059.[PMID: 15742415]
http://dx.doi.org/10.3748/wjg.v11.i7.1056

[46] Jaattela M, Wissing D, Kokholm K, Kallunki T, and Egeblad M. Hsp70 exerts its anti apoptotic function downstream of caspase 3 like protect .*The EMBO Journal* 1998;17:6124–6134.[PMID: 9799222]
http://dx.doi.org/10.1093/emboj/17.21.6124

[47] Mosser D, Caron AW, Bourget L, Denis-Larose C, and Massie B. Role of the human heat shock protein hsp70 in protection against stress induced apopotosis. *Molecular and Cellular Biology* 1997;17:5317-5327.[PMID: 9271409]
http://dx.doi.org/10.1128/MCB.17.9.5317
[48] Takashima M, Kuramitsu Y, Yokoyama Y, Lizuka N, Toda T. Proteomic profiling of heat shock protein 70 family members as biomarkers for hepatitis C virus-related hepatocellular carcinoma. *Poteomics* 2003;3:2487-493.[PMID: 14673798]
http://dx.doi.org/10.1002/pmic.200300621
[49] Zhao ZG, and Shen WL. Heat shock protein 70 antisense oligonucleotide inhibits cell growth and induces apoptosis in human gastric cancer cell line SGC-7901. *World Journal of Gastroenterology* 2005;11:73-78.[PMID: 15609400]
http://dx.doi.org/10.3748/wjg.v11.i1.73
[50] Rendon-Huerta E, Mendoza-Hernadez G, and Robles-Flores M. Characterization of calreticulin as a protein interacting with protein kinase C. *Biochemistry Journal* 1999;344:469-475.[PMID: 10567230]
http://dx.doi.org/10.1042/bj3440469
http://dx.doi.org/10.1042/0264-6021:3440469
[51] Mesaeli N, and Phillipson C. Impaired p53 expression, function and nuclear localization in calreticulin deficient cells. *Molecular Biology of the Cell* 2004;15:1862-1870.[PMID: 14767071]
http://dx.doi.org/10.1091/mbc.E03-04-0251
[52] Trask DK, Band V, Zajchowski DA, Yaswen P, Suh T, and Sager R. Keratins as markers that distinguish normal and tumor derived mammary epithelia cells. *PNAS* 1990;87:2319-2323.[PMID: 1690428]
http://dx.doi.org/10.1073/pnas.87.6.2319
[53] Kinjo T, Kamiyama K, Chinen K, Iwamasa T, Kurihara K, Hamada T. Squamous metaplasia induced by transfection of human papillomavirus DNA into cultured adenocarcinoma. *cells.Journal of Clinical Pathology* 2003;56:97-108.[PMID: 12665627]
http://dx.doi.org/10.1136/mp.56.2.97
[54] Yamamoto K, Oka M, Hayashi H, Tangoku A, Gondo T, Suzuki T. CYFRA 21-1 is a useful marker for esophageal squamous cell carcinoma. *Cancer* 1997;79:1647-1655.[PMID: 9128978]
http://dx.doi.org/10.1002/(SICI)1097-0142(19970501)79:9<1647::AID-CNCR3>3.0.CO;2-9
http://dx.doi.org/10.1002/(SICI)1097-0142(19970501)79:9<1647::AID-CNCR3>3.3.CO;2-F
[55] Ladanyi M. Aberrant ALK tyrosine kinase signaling, Different Cellular Lineages, Common Oncogenic Mechanisms. *American Journal of Pathology* 2000;157:341–345.[PMID: 10934137]
http://dx.doi.org/10.1016/S0002-9440(10)64545-2
[56] Lamant L, Dastuque N, Pulford K, Delsol G, and Mariame B. A new fusion gene *TPM3-ALK* in anaplastic large cell lymphoma created by a (1;2)(q25;p23) translocation. *Blood* 1999;93:3088–3095.[PMID: 10216106]
[57] Lawrence B. Perez-Atayde, A. Hibbard MK, Rubin BP, Dal Cin P, Pinkus JL,Pinkus GS, Xiao S, Yi ES, Fletcher CD, Fletcher JA. .TPM3-ALK and TPM4-ALK oncogenes in inflammatory myofibroblastic tumors. *American Journal of Pathology* 2000;157:377-384.[PMID: 10934142]
http://dx.doi.org/10.1016/S0002-9440(10)64550-6