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Proteomic Identification of Malignant Transformation-Related Proteins in Esophageal Squamous Cell Carcinoma

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Abstract Esophageal cancer (EC) persists to be a leading cancer-related death in northern China. Clinical outcome of EC is the most dismal among many types of digestive tumors because EC at early stage is asymptomatic. The current study used 2-DE-based proteomics to identify differentially expressed proteins between esophageal cancer cell lines and immortal cell line. Fifteen proteins were identified with differences of more than five folds, comprising the down-regulation of annexin A2, histone deacetylase 10 isoform beta and protein disulfide-isomerase ER-60 precursor, and the up-regulation of heat shock 70 kDa protein 9B precursor, solute carrier family 44 Member 3, heterogeneous nuclear ribonucleoprotein L (hnRNP L), eukaryotic translation initiation factor 4A isoform 2, triosephosphate isomerase1 (TPI), peroxiredoxin1 (PRX1), forminotransferase cyclodeaminase form (FTCD), fibrinogen gamma-A chain precursor, kinesin-like DNA binding protein, lamin A/C, cyclophilin A (CypA), and transcription factor MTSG1. Expression pattern of annexin A2 was verified by Western blotting, immunocytochemistry and immunohistochemistry analysis. The implication of these protein alterations correlated to the esophageal malignant transformation is discussed. J. Cell. Biochem. 104: 1625–1635, 2008. © 2008 Wiley-Liss, Inc.

Key words: esophageal squamous cell carcinoma; proteomics; protein profiling; malignant transformation-associated proteins; annexin A2

Esophageal squamous cell carcinoma (ESCC), the main histological subtype of esophageal cancer (EC), persists to be a leading cancer-related death in northern China [Yang, 1980; Wang et al., 1997; Lightdale, 1999] Although a variety of oncogenes, tumor suppressor genes, apoptosis-associated genes, metastasis-related genes [Yoshida et al., 1990; Jones et al., 1993; Wong et al., 1994; Shi et al., 1999; Xing et al., 1999b; Cai et al., 2000] as well as epigenetic abnormalities, for example, methylation, acetylation, etc. [Xing et al., 1999a; Jones and Takai, 2001; Nie et al., 2001, 2002; Esteller, 2002; Jones and Baylin, 2002; Wang et al., 2003], have been found to play crucial roles during the development and progression of esophageal cancer, the high mortality rate of ESCC is still prevalent due to diagnosis at advanced stages at which modern therapeutic modalities always succumb. Ninety percent of esophageal cancer patients can survive more than 5 years if the cancer is

Abbreviations used: ESCC, esophageal squamous cell carcinoma; ICC, immunocytochemistry; IHC, immunohistochemistry; TPI, triosephosphate isomerase1; PRX1, peroxiredoxin1; FTCD, forminotransferse cyclodeaminase form; CypA, cyclophilin A; hnRNP L, heterogeneous nuclear ribonucleoprotein L.

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detected at an earlier stage [Wang, 2001]. Evidently, identification of aberrant molecules at the earliest stage of esophageal carcinogenesis holds the bottleneck of early detection and diagnosis as well as the therapeutic management of esophageal cancer.

It is well known that development of ESCC from normal esophageal epithelium proceeds a multi-stage process with stepwise accumulation of genetic and/or epigenetic defects. As one of critical prerequisites of malignant transformation, immortalization may represent the earliest stage for ESCC [Xiaoxue et al., 2004]. Distinct molecules between immortal cells and malignant transformed cells would be suitable candidate biomarkers for early diagnosis and for understanding the insights underlying molecular carcinogenesis of esophageal cancer.

Proteomics has been extensively applied to characterize the protein repertoires of one type of cell, tissue or organism at a given time point. By using 2-DE-based proteomic profiling, we have previously analyzed tumor entity of ESCC and nearby non-cancer epithelium, and identified a large body of differentially expressed proteins associated with esophageal pathogenesis [Qi et al., 2005]. In order to overcome the heterogeneity of tissue samples, we used immortal cell lines in present study. Differential expression of proteins between immortal cell line NECA-E6E7-hTERT and three tumorigenic cell lines EC1, EC18 and EC109 was analyzed by proteomic approach with an aim to identify proteins associated with malignant transformation of esophageal epithelium. Subsequent validation on tumor samples by annexin A2 immunostaining was also performed. The current findings may help us to understand the malignant transformation and provide more candidate biomarkers for ESCC early detection and diagnosis.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Three esophageal cancer cell lines, EC1, EC18 and EC109, were grown in RPMI1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO) at 37°C with 5% CO_2 in atmosphere. The cancer cells were trypsinized when 80% confluence was reached and reseeded at 1:8 split ratio. Immortal esophageal cell line NECA-E6E7-hTERT was established by transferring HPV-E6E7 and catalytic protein subunit of telomerase (hTERT) into normal esophageal epithelium nearby esophageal cancer lumps, which had been confirmed by pathological examination [Morales et al., 2003]. All of the cell lines were generous gifts from Prof. George Tsao, Department of Anatomy at the University of Hong Kong. NECA-E6E7-hTERT was cultured in keratinocyte-serum-free-medium (KSFM, GIBCO) supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE). The immortal cells at 80% confluence were treated with EDTA (GIBCO) followed by 0.05% trypsin treatment when cells show round and then passaged at 1:8 split ratio.

Tissue Samples

Tissues used in this experiment were obtained with the approval of the Committees for Ethical Review of Research involving Human Subjects at Henan University and the University of Hong Kong. A total of 33 primary esophageal squamous cell carcinoma samples together with nearby normal esophageal epithelium were collected from Henan, China, one of highest area for EC incidence. The samples were maintained in liquid nitrogen or deep-freezer (−80°C) until use. Cancer and normal epithelium tissues were dissected from tumor lump and nearby epithelium at least 5 cm distal to tumor lump, respectively. The presence of cancer cells and normal esophageal epithelium for each sample was confirmed histopathologically by two independent pathologists' examination.

Soft Agar Cloning

Soft agar cloning assay was used to test the tumorigenity of esophageal cancer cell lines EC1, EC18, EC109 and immortal cell line NECA-E6E7-hTERT with CNE (lung cancer cell) as a positive control. A 0.6% agar/medium was prepared by mixing 1.2% agar (w/v) at 60°C and 2× RPMI1640 (GIBCO) for three esophageal cancer cell lines or 2× DMEM (GIBCO) for immortal cell line with 10% bovine calf serum. One milliliter of 0.6% agar/medium was applied into each well of a 6-well plate and allowed to gel as a bottom layer. Immortal cell line NECA-E6E7-hTERT was cultured in DMEM for 3 weeks for accustomization prior to soft agar analysis. Cells were trypsinized and resuspended at dilutions of 2×10^5, 2×10^4,

2×10^3 per ml of DMEM. A mixture of 1 ml of 0.6% agar/medium and 1 ml of cell suspensions at different dilutions were overlaid onto the bottom layer of gel. The 0.3% agar/medium-containing cells were allowed to solidify at room temperature and then incubated at 37°C in a humified 5% CO_2/95% air atmosphere. Growth of cells was maintained by weekly overlaying 250 μl of RPMI1640 or DMEM over gel supplemented with 10% fetal calf serum. Colony formation was evaluated under microscope after 3 weeks incubation.

Cell Harvest and Protein Extraction

Upon reaching 80% confluent growth, cells in 100 mm culture plates were harvested by using a rubber scraper. The harvested cells were rinsed with ice-cold PBS and centrifuged at 1,000 rpm for 5 min followed by discarding the supernatant. The washing procedure was performed twice to remove residual culture medium. The final cell pellets were stored at −80°C until further use. As for protein extraction, the cell pellets were lysed in lysis buffer (8 M urea, 4% CHAPS, 2% Pharmalyte) and allowed to stand on ice for 30 min. The lysate was centrifuged at $16 \times 1{,}000g$ at 4°C for 5 min to remove cell debris. The supernatant was taken as extracted proteins and the protein concentration was determined by the Bradford method with BSA as standard. Aliquots of protein samples were kept as cell pellets.

2-DE, Image Analysis, and MS

2-DE, silver staining and image analysis were carried out as previously described [Qi et al., 2005]. Briefly, the first dimension of IEF was conducted on a linear pH range of 3–10 using Amersham Biosciences IPGphor. Second dimension separation of proteins by mass was completed by using 15% sodium dodecyl sulphate–polyAcrylamide gel electrophoresis (SDS–PAGE). Image acquisition and analyses were accomplished with ImageScanner (Amersham Biosciences) and ImageMaster 2-D Elite software 4.01 (Amersham Biosciences), respectively. The normalized volume for each protein spot was used for comparison. All samples were run at least in duplicate to guarantee reproducibility. Spots of interest were excised with a clean scalpel from the preparative gels and in-gel digestion was subsequently performed. Peptide mass spectra were recorded and parameters for spectral acquisition were used as stated previously [He et al., 2003]. Proteins were identified by peptide mass fingerprinting using MS-Fit to search the NCBInr protein database (http://prospector.ucsf.edu). The criteria for database matching are ±25 ppm mass tolerance, one missed cleavage allowance, at least four peptides matched and corresponding molecular weights and pI values. The species of origin was restricted to Homo sapiens.

Western Blotting

With reference to the verification of candidate proteins after peptide mass fingerprinting, proteins of interest were selected for Western blotting to confirm the protein identification. After SDS–PAGE, proteins were transferred onto PVDF membranes (Amersham Biosciences) at 0.8 mA/cm^2 for 1 h. After blocking in 5% non-fat milk in TBS-T containing 0.1% Tween 20 (Sigma) at 4°C overnight with gentle rocking, membranes were probed with primary antibody against annexin A2 at dilution of 1:200 (Santa Cruz). After incubation with HRP-conjugated secondary antibody against rabbit IgG, immunoblots were visualized with the ECL detection kit (Amersham Biosciences). After development and exposure, the membrane was stripped with stripping buffer (glycine 3.75 g/L, SDS 2 g/L, pH 2.0) before blocking followed by TBST washing for 5 min twice and then incubated with antibody against β-actin for loading equalization.

Immunocytochemistry (ICC) and Immunohistochemistry (IHC) for Annexin A2

With regard to ICC, cells were allowed to grow on cover slides in dishes for 4 days at 37°C with 5% CO_2. The cells on cover slide were fixed with 85% ethanol before dehydration. Tissue samples of esophageal squamous cell carcinoma and nearby normal esophageal epithelium were fixed in 4% paramaldehyde, hydrated in ethanol gradient, permeabilized in xylene and embedded in paraffin. Tissue sections of 5 μm were cut for hematoxylin and eosin staining and annexin A2 immunostaining. After dewaxing in xylene, slides mounted with tissue sections and EC1, EC18, EC109 esophageal cancer cells were dehydrated in ethanol gradient and subjected to PBS washing for 5 min twice. Incubation with 0.3% H_2O_2 was used to quench endogenous peroxide for 20 min at room temperature and with 2% normal rabbit serum to block non-

specific reaction for 20 min. The sections were incubated with annexin A2 diluted to 1:200 at 4°C overnight and then with anti-rabbit secondary antibody for 1 h at room temperature. Avidin–biotin complex kit and diaminobenzidine kit was used to visualize antigen-antibody complex. Substitution of PBS for annexin A2 antibody was used for negative control. Multiplication product (0–9) of immunostaining intensity (0–3) and percentage of immunopositive cells (0–3) was evaluated by two independent pathologists as the final staining scores. Consensus was reached whenever disagreement occurred.

Statistical Analysis

Statistical analysis was performed using two-tailed Student's t-test, and $P < 0.05$ was considered significant. Data were expressed as the mean ± SD of triplicate samples, and reproducibility was confirmed in three separate experiments.

RESULTS

Soft Agar Analysis

The capability of cultured cells growing in soft agar (anchorage independent) is closely correlated with its tumorigenicity in immune deficient animals such as nude mice [Hahn et al., 1999; Morales et al., 2003]. The colony image of four cell lines was shown in Figure 1. The cloning rates of esophageal cancer cell lines EC1, EC18 and EC109, and immortal cell line NECA-E6E7-hTERT were shown in Table I. EC109 cancer cell shows the highest cloning rate followed by EC1 and EC18. As expected, NECA-E6E7-hTERT did not develop any colony in soft agar culture, which corresponds to its non-malignant phenotype.

2-DE Image Analysis and Protein Identification by PMF

On a typical 13 cm × 16 cm 2-D gel image, around 1,200 protein spots were detected for esophageal cancer cell lines and immortal cell line after editing by ImageMaster 2-D Elite software. The averaged normalized volumes of each protein spot from EC1, EC18, EC109 cancer cells were used for comparison with those of the corresponding spot from immortal cells. A total of 56 protein spots consistently showed more than twofold differential expression with 30 protein expression increased and 26 decreased in esophageal cancer cells. When fivefold difference cut-off was used, 17 protein spots were found to be consistently augmented

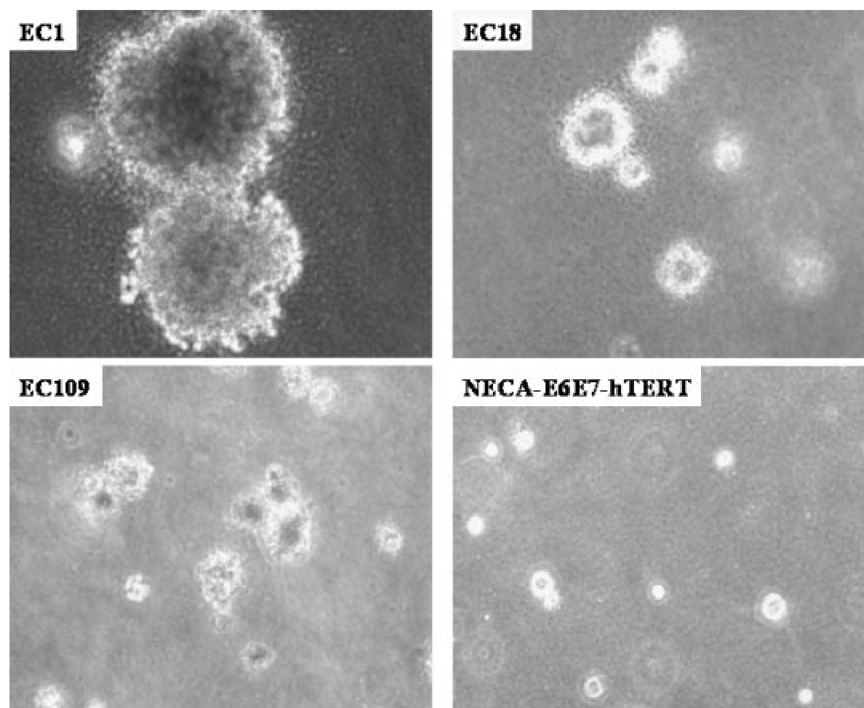

Fig. 1. Anchorage independent growth of esophageal cancer cells EC1, EC18, EC109 and immortal esophageal epithelial cells NECA-E6E7-hTERT.

TABLE I. Cloning Efficiency of Esophageal Cancer Cell Lines and Immortal Cell Line

Cell lines	Cloning efficiency
CNE1 (positive control)	39.8 ± 7.4
EC1	19.0 ± 4.3
EC18	14.5 ± 3.0
EC109	33.6 ± 6.2
NECA-E6E7-hTERT	0

and 13 declined in cancer cells. Figure 2 shows spot alterations for four typical protein spots with spot number of 994, 1,184, 1,236, and 2,148 in EC1, EC18, EC109, and NECA-E6E7-hTERT cells.

Among the 30 protein spots with fivefold differences in expression between esophageal cancer cell and immortal cell, spots with sufficient protein amounts (based on volume intensity) were excised and subjected to in-gel digestion, MALDI-TOF mass spectral analysis, and database searching for protein identification. Fifteen protein spots were found to produce high-quality mass spectra that met the restrict database-matching criteria for protein identification. Table II shows the protein ID, spot numbers, theoretical and experimental molecular weights and pI, percentage of peptides matched, sequence coverage, total mass error, MOWSE score as well as difference folds. In three down-regulated proteins, annexin A2 shows the largest under-expression (13.4 folds) in esophageal cancer cells. Among the 12 up-regulated proteins, expression of heterogeneous nuclear ribonucleoprotein L augments about 29.4 folds.

Western Blotting, ICC and IHC

Since Annexin A2 has been implicated to play a role in a variety of human tumors originating from different tissues or organs, including lung, bone, colon, stomach, liver, breast, and buccal mucosa [Cole et al., 1992; Vishwanatha et al., 1993; Menell et al., 1999; Chetcuti et al., 2001; Emoto et al., 2001a,b; Wulfkuhle et al., 2002; Chen et al., 2004; Gillette et al., 2004; Katayama et al., 2006], it was selected for Western blotting, ICC and IHC analyses to validate the protein expression patterns in cells and tissues. Figure 3 shows the Western blotting results

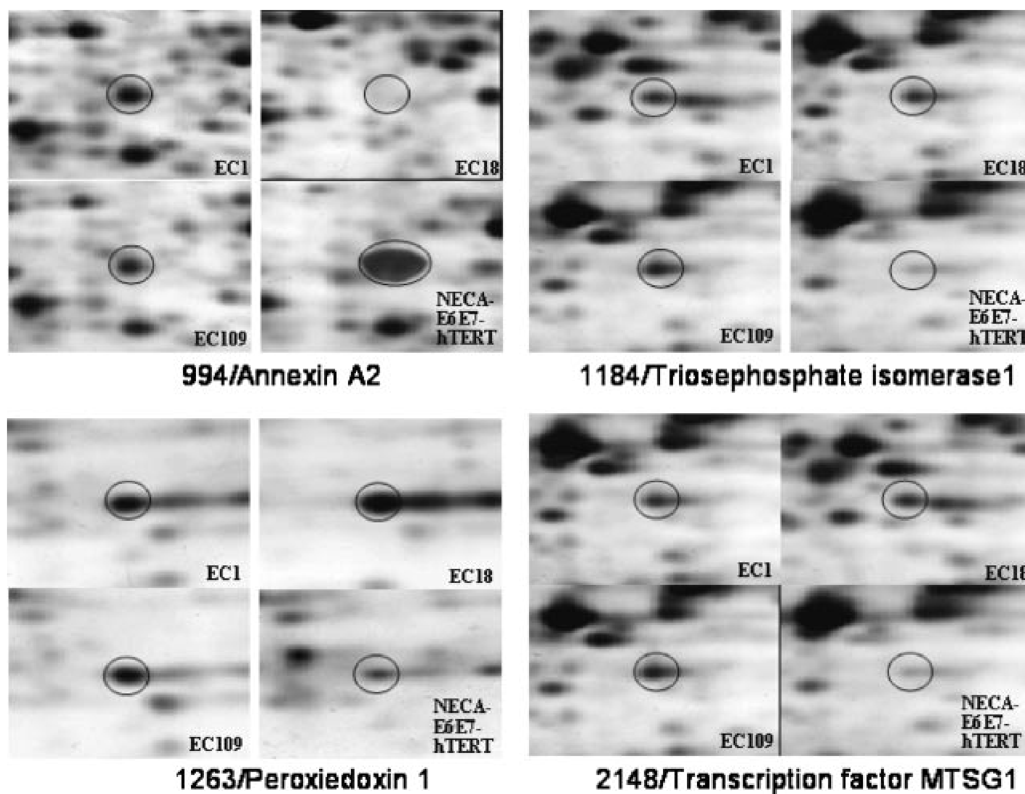

Fig. 2. Cropped images of protein spots 994, 1,184, 1,263, and 2,148 for esophageal cancer cells EC1, EC18, EC109 and immortal esophageal epithelial cells NECA-E6E7-hTERT derived from 2-DE gel images.

TABLE II. Differentially Expressed Proteins in Cancer Cell Lines and Immortal Cell Line of Human Esophagus

Spot no.	Protein ID (MW/pI)	Experimental mass (kDa)/pI	Peptides matched (%)	Sequence coverage (%)	Total mass accuracy	MOWSE score	Difference folds
398	Heat shock 70 kDa protein 9B precursor 73681/5.9	75.0/5.5–6.0	9	13.0	11.4	3.13E+02	+5.2 ± 1.2
423	Solute carrier family 44 Member 3 68047/7.7	65.0/6.0–6.5	13	19.3	16	608	+5.1 ± 0.8
449	Heterogeneous nuclear ribonucleoprotein L 60188/6.7	60.0/6.0–6.5	21	18.5	24	2.25E+05	+29.4 ± 3.6
839	Eukaryotic translation initiation factor 4A, isoform 2 46736/5.3	50.0/4.5–5.0	20	10.6	7.23	591	+25.6 ± 3.1
994	Annexin A2 38576/7.6	40.0/7.0–7.5	71	27.1	7.13	5.79E+04	−13.4 ± 1.9
1,084	Protein disulfide-isomerase ER-60 precursor 6783/6.0	46.0/6.0–6.5	23	15.8	23.4	1.17E+04	−8.1 ± 2.2
1,184	Triosephosphate isomerase1 26642/6.4	26.5/6.5–7.0	41	22.9	11.6	209	+5.1 ± 1.5
1,263	Peroxiredoxin1 22111/8.3	24.0/7.5–8.0	17	51	16.4	7.732E+06	+5.1 ± 2.0
1,274	Forminotransferase cyclodeaminase form 52766/5.9	50.0/5.5–6.0	36	19.3	43.3	748	+5.79 ± 1.8
1,300	Fibrinogen gamma-A chain precursor 49497/5.7	50.0/5.5–6.0	69	19.8	35.6	243	+12.8 ± 2.5
1,440	Histone deacetylase 10 isoform beta 69415/5.7	15.0/5.5–6.0	7	17.9	13.6	1.12E+02	−9.3 ± 2.8
1,859	Kinesin-like DNA binding protein 72376/9.5	65.0/9.0–9.5	18	15.7	24.5	436	+14.9 ± 3.3
1,889	Lamin A/C 65135/6.4	60.0/6.0–6.5	6	17.7	23.4	2.12E+6	+13.6 ± 2.8
1,973	Cyclophilin 17972/7.7	20.0/7.5–8.0	69	15.5	30.8	6.74E+03	+5.9 ± 2.5
2,148	Transcription factor MTSG1 50544/8.5	46.0/8.0–8.5	48	17.7	37.7	234	+5.2 ± 1.7

for annexin A2 in three esophageal cancer cells and immortal cells revealing that immortal esophageal cell NECA-E6E7-hTERT expressed annexin A2 protein much higher than EC1, EC18 and EC109. Using cells grown on cover slides, we performed ICC to see the location and expression of annexin A2 protein in different types of cells. From annexin A2 immunostaining on NECA-E6E7-hTERT cells in Figure 4, annexin A2 located mainly in nucleus, rarely in cytoplasm and membrane. Only a few cells expressed annexin A2 protein moderately in EC1, EC18, and EC109 cells. Interestingly, annexin A2 protein prominently located in cytoplasm and/or cell membrane on tissue sections of esophageal squamous cell cancer and nearby normal epithelium as shown in Figure 5. Further analysis found that, in the normal epithelium, annexin A2 presented mainly in the cytoplasm of basal cells, which adhere to basal membrane; and in parabasal cells and upper layer cells, annexin A2 migrated to cell membrane and scarcely presented in cytoplasm. In addition, annexin A2 protein expression was down-regulated stepwise when epithelial cell was transformed malignantly (Fig. 5). In poorly differentiated squamous carcinoma, 46% (5/11) of cancer tissue sample lost annexin A2 protein totally and 36% (4/11) expressed at low extent as shown in Table III.

DISCUSSION

Most of esophageal cancer patients were detected and diagnosed at an advanced stage at present. Ninety percent late-stage esophageal cancer patients cannot survive more than 5 years despite of surgical treatment in

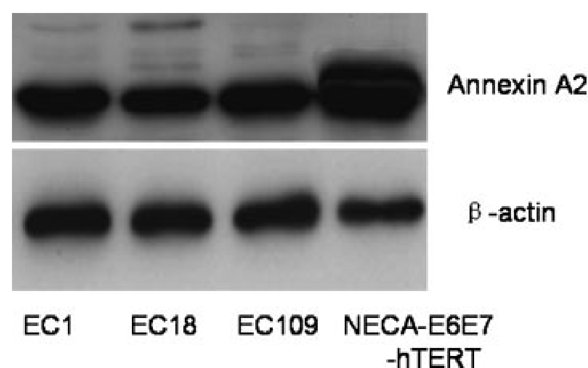

Fig. 3. Western blotting analysis of annexin A2 expression in esophageal cancer cells EC1, EC18, EC109 and immortal esophageal epithelial cells NECA-E6E7-hTERT.

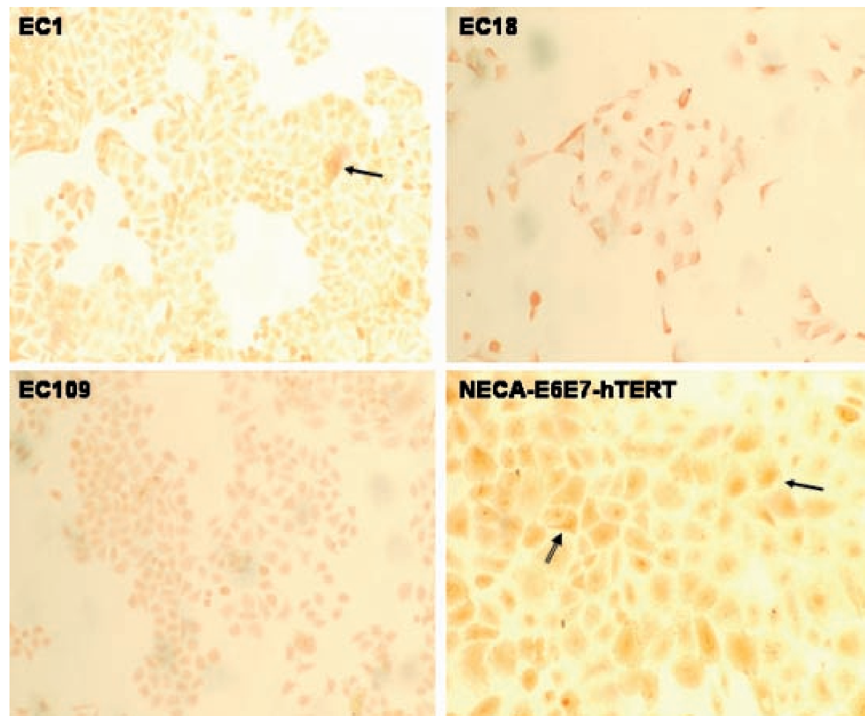

Fig. 4. Annexin A2 immunostaining on esophageal cancer cells EC1, EC18, EC109 and immortal esophageal epithelial cells NECA-E6E7-hTERT growing on cover slides. Arrow indicates strong expression of annexin A2 inside cells.

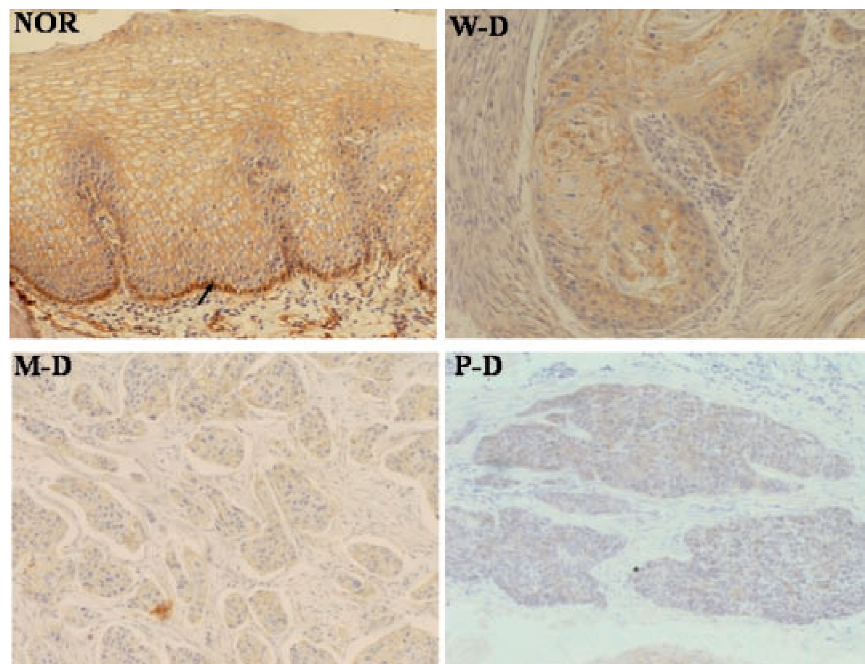

Fig. 5. Immunohistochemistry analysis of annexin A2 staining on normal esophageal epithelial (NOR), well-differentiated ESCC (W-D), moderately differentiated ESCC (M-D) and poorly differentiated ESCC (P-D).

TABLE III. Immunohistochemistry Staining Intensity of Annexin II in Esophageal Squamous Cancer and Adjacent Normal Epithelium

Histology	Case no.	Intensity			
		0	+	++	+++
Normal[a]	33	3 (9)	0	18 (55)	12 (36)
Well-differentiated SCC	7	0	4 (57)	2 (29)	1 (14)
Moderately differentiated SCC[a]	15	4 (34)	5 (45)	3 (20)	3 (20)
Poorly differentiated SCC[a]	11	5 (46)	4 (36)	2 (18)	0

[a]Statistical significance ($P < 0.01$) between normal and moderately differentiated SCC and normal and poorly differentiated SCC.

combination with radiotherapy and chemotherapy [Oka et al., 1996]. In contrast, esophageal cancer cases diagnosed histopathologically as carcinoma in situ or itramucosal carcinoma survived more than 5 years only after musocal resection under endoscopy guidance [Wang, 2001].

Precursors of esophageal cancer, such as basal cell hyperplasia and dysplasia, are unstable and able to regress or progress but the mechanisms remain elusive. Biomarkers related to the progression of precursors for esophageal carcinoma are suitable for early diagnosis and for monitoring high-risk population, and thus are highly probable to reduce incidence of esophageal cancer. As one of the most important features of malignant cells, immortalization precedes malignant transformation and thus may resemble the precursors of esophageal carcinoma [Xiaoxue et al., 2004]. Identification of distinct molecules between immortal cells and cancer cells may provide useful candidate proteins for evaluation as the potential biomarkers for early-stage diagnosis of EC. Further characterization of these candidate proteins may also help us to better understand the pathogenesis of EC malignant transformation. With these hypotheses in mind, we compared the protein profiles between immortal esophageal cells and esophageal cancer cells by proteomic approach and identified a group of proteins with differential expression for validation and analysis. Multiple cancer cell lines were used to confirm the consistency of the dysregulation of the proteins identified.

One of the most prominent changes in protein expression was annexin A2; the protein was down-regulated more than 13 folds in malignant esophageal cancer cells. It suggests that loss of annexin A2 protein may contribute to the development and progression of esophageal carcinoma. As a member of annexin family of Ca^{2+}-dependent phosphlipid and membrane binding, annexin A2 functions as a substrate for receptor and nonreceptor protein kinases. This protein has been implicated in cell proliferation, differentiation, inflammation, endocytosis, exocytosis, membrane fusion, membrane/cytoskeletal interations, and membrane channel activation [Creutz, 1992; Harder et al., 1997; Faure et al., 2002; van de Graaf et al., 2003]. In one study, annexin A2 has been found to get lost 100% in prostate cancer (31/31) and 65% in high-grade prostate intraepithelial neoplasia (PIN) [Chetcuti et al., 2001]. Another study revealed that annexin A2 expression was decreased in human osteosarcoma (OS) metastases and metastatic cell lines at both mRNA and protein levels [Gillette et al., 2004]. Overexpression of annexin A2 in metastatic variant of OS cancer cell reduced the potential of osteosarcoma cells to form primary tumors and to develop colonies following intravenous inoculation in mice with no effects on cell proliferation and cell cycle.

In contrast, some investigations showed positive roles of annexin A2 in tumor development and progression, such as in primary primitive neuroectodermal tumors, liver cancer, colorectal cancer, lung cancer, acute promyelocytic leukemia, stomach cancer, pancreatic cancer, etc. [Cole et al., 1992; Vishwanatha et al., 1993; Menell et al., 1999; Emoto et al., 2001a,b; Wulfkuhle et al., 2002]. Our previous proteomic study also found that annexin A2 protein was enhanced 4.8 folds in buccal squamous cell carcinoma [Chen et al., 2004]. These collective research results suggested that distinct roles of annexin A2 in different type of tumors may reflect tumor origin and tissue specificity. Considering the distinctive expression pattern of annexin A2 in various tumors, the specificity of

annexin A2 in ESCC may be worthy for further evaluation as a potential biomarker. The physiological functions of annexin A2 in normal esophageal epithelium and its roles during multi-stage esophageal carcinogenesis are under further investigation.

In addition to annexin A2, other 14 proteins including histone deacetylase 10 isoform beta, protein disulfide-isomerase ER-60 precursor, heat shock 70 kDa protein 9B precursor, solute carrier family 44 Member 3, hnRNP L, eukaryotic translation initiation factor 4A isoform 2, triosephosphate isomerase1 (TPI), peroxiredoxin1 (PRX1), forminotransferase cyclodeaminase form (FTCD), fibrinogen gamma-A chain precursor, kinesin-like DNA binding protein, lamin A/C, cyclophilin A (CypA) and transcription factor MTSG1 were found to have more than fivefold differences in expression between immortal and malignant phenotypes, suggesting that other physiological processes including energy metabolism, intracellular signal transduction, translation, pre-mRNA processing, cell defensive and chaperone activity were involved in ESCC malignant transformation.

Produced in cells during physiological processes, reactive oxygen species (ROS) plays important roles in initiation, promotion and progression of neoplastic pathogenesis [Klaunig et al., 1998]. To maintain redox balance, cells have evolved antioxidant systems, for example, protein superoxide dismutase (SOD), catalase, glutathione peroxidase and peroxiredoxins (PRX). The increased expression of peroxiredoxin 1 and decreased expression of protein disulfide-isomerase ER-60 precursor found in the present study implicate that the cellular redox balance was interrupted in transformed malignant esophageal cells. Our previous study with ESCC tissues also revealed that expression of Mn-SOD and PRX1 were up-regulated in esophageal cancer while PRX2 was down-regulated in comparison with nearby normal epithelium [Qi et al., 2005]. Distinct expression pattern of anti-oxidant proteins or different isoforms may reflect varied functions of each component in the antioxidant system.

TPI is an enzyme which catalyzes the conversion of dihydroxyacetone phosphate to glyceraldehydes 3-phosphate in glycolytic pathway. The aberrant increase of TPI expression in ESCC corresponds to the high level of metabolic requirement by rapid growth of cancer cells. In bladder and colon cancers, TPI displayed increased expression as well, and particularly in lung cancer, TPI showed significantly higher expression at more advanced stages [Montgomerie et al., 1997; Katayama et al., 2006]. Using colon cancer cell lines SW480 and SW620 that derived from primary lesions and metastatic lymph node, respectively, a proteomic study revealed that TPI expression was prominently enhanced in SW620 [Katayama et al., 2006]. More intrigingly, TPI, together with other five glycolytic enzymes, was present in serum of xenograft mouse of human prostate cancer.

CypA is a member of cyclophilin protein family which manifests many biological properties and functions, including peptidyl-prolyl *cis–trans* isomerase activity, protein trafficking, regulation of T-cell function and inflammation, maintenance of mitochondrial functions and involvement of apoptosis [Ivery, 2000; Huang et al., 2002]. In addition, CypA mediates the action of the immunosuppressive drug, cyclosporine A [Handschumacher et al., 1984]. Recent investigations indicated possible roles of CypA in the formation and development of cancer [Rey et al., 1999; Campa et al., 2003]. Consistent with our present finding of the drastic increase in CypA expression, CypA was found significantly high expressed in pancreatic cancer cell lines and tissues. Pancreatic cancer cells treated with exogenous CypA showed remarkably high proliferation in a dose-dependent fashion and this effect was blocked by pre-treatment with an anti-CD 147 antibody [Li et al., 2006]. Stable RNA interference-mediated knock-down of CypA in two non-small-cell lung cancer cell lines led to reduced growth, less fluorodeoxyglucose uptake, decreased proliferation and a greater degree of apoptosis when the cells with knock-down of CypA were grown as xenografts in severe immunodeficient mice [Howard et al., 2005].

hnRNP L was identified as a main nuclear protein which has three RNA recognition motifs, several glycine- and proline-rich regions [Pinol-Roma et al., 1989]. Previous studies revealed that hnRNP L can make effects on the translation of hepatitis C virus and the stability of vascular endothelial growth factor and glucose transporter1 mRNA. hnRNP L also enhances the stability and stimulates the splicing of endothelial cell nitric oxide synthase pre-mRNA by binding to variable-length CA

repeats [Hui et al., 2003a,b]. A more recent study found that binding of hnRNP L was able to increase stability, ployadenylation and cytoplasmic accumulation of transcripts synthesized in CV-1 cells from an intronless variant of the human β-globin gene when present in two or more tandem copies [Guang et al., 2005]. The dramatic increase of hnRNP L expression in the present ESCC may reflect the involvement of the pre-mRNA processing in the tumorigenesis.

In conclusion, a number of proteins were identified with expression differences of more than five folds between esophageal cancer cells and immortal cells by 2-DE based proteomic technology. The altered expression pattern of annexin A2 was verified by Western blotting and immunocytochemistry and was further validated by immunohistochemistry analysis in esophageal tissues. The significant changes of other proteins in expression implicate the involvement of many pathways in the ESCC malignant transformation. The possibility of these altered proteins to be potential biomarkers for high-risk esophageal cancer screening warrants further evaluation.

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