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DIFFERENTIALLY EXPRESSED PROTEINS BETWEEN ESOPHAGEAL SQUAMOUS CELL CARCINOMA AND ADJACENT NORMAL ESOPHAGEAL TISSUE

PROTEINI SA RAZLIČITOM EKSPRESIJOM U PLANOCELULARNOM KARCINOMU JEDNJAKA I TKIVU SUSEDNIH NORMALNIH ĆELIJA JEDNJAKA

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Summary: Proteomics was employed to identify the differentially expressed proteins between esophageal squamous cell carcinoma (ESCC) and adjacent normal esophageal tissues. ESCC tissues and adjacent normal tissues were obtained from 10 patients with ESCC and the proteins were extracted and subjected to two-dimensional gel electrophoresis (2-DE). The differentially expressed proteins were identified after image analysis, and matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) was used to confirm these proteins. Immunohistochemistry was then performed to detect the expressions of HSP27 and ANX1 in ESCC tissues and adjacent normal tissues. A total of 6 differentially expressed proteins were identified by peptide mass fingerprinting, among which SCCA1, KRT4 and ANX1 were down-regulated and TIM1, MnSOD and HSP27 up-regulated in the ESCC. Immunohistochemistry showed HSP27 was highly expressed in the ESCC which, however, had a low expression of ANX1. These findings were consistent with those in proteomics. There were differentially expressed proteins between ESCC and adjacent normal tissues. The investigation of differentially expressed proteins between ESCC and normal esophageal tissue may provide evidence for the molecular pathogenesis of ESCC.

Keywords: esophageal squamous cell carcinoma, proteomics, differentially expressed proteins, immunohistochemistry

Kratak sadržaj: Uz pomoć proteomike, identifikovali smo proteine čija se ekspresija razlikuje u planocelularnom karcinomu jednjaka (PKJ) i susednim zdravim tkivima jednjaka. Uzorci tkiva PKJ i susednih normalnih tkiva uzeti su od 10 pacijenata sa PKJ a proteini su ekstrahovani i podvrgnuti dvodimenzionalnoj elektroforezi na gelu (2-DE). Proteini sa različitom ekspresijom su identifikovani posle analize slika, dok je za njihovo potvrđivanje upotrebljena tehnika MALDI-TOF-MS (*matrix-assisted laser desorption ionisation time-of-flight mass spectrometry*). Potom su obavljene imunohistohemijske analize kako bi se utvrdile ekspresije HSP27 i ANX1 u tkivima PKJ i susednim normalnim tkivima. Pomoću tehnike *peptide mass fingerprintinga* identifikovano je ukupno šest proteina sa različitim ekspresijama, od kojih je ekspresija SCCA1, KRT4 i ANX1 bila snižena, a TIM1, MnSOD i HSP21 povišena u PKJ. Imunohistohemijsko ispitivanje je pokazalo da je HSP27 veoma izražen u PKJ, u kom je, međutim, utvrđena niska ekspresija ANX1. Ovi rezultati se slažu sa onima dobijenim pomoću proteomike. Utvrđeno je da postoje proteini sa različitom ekspresijom u PKJ i susednim normalnim tkivima. Istraživanje različite ekspresije proteina u PKJ i normalnom tkivu jednjaka može doneti nove dokaze u vezi s molekulskom patogenezom PKJ.

Ključne reči: planocelularni karcinom jednjaka, proteomika, proteini s različitom ekspresijom, imunohistohemija

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Introduction

Esophageal squamous cell carcinoma (ESCC), the predominant histological subtype of esophageal cancer, is the fourth most common malignancy. The aggressive behavior of this tumor is often associated with systemic spread of the disease at the time of

diagnosis (1). The incidence of ESCC is relatively high in China. The pathogenesis of ESCC has been found to be associated with some factors, although the exact pathogenesis is still unknown (2). With the development and improvement of proteomics, cancer proteomics has been used as a useful tool in numerous studies on cancers (3). The investigations on the differentially expressed proteins between cancer tissues and normal tissues may be beneficial for understanding the mechanisms underlying the pathogenesis and development of the cancers, and helpful to find cancerogenesis-related proteins and markers of cancers (4). In the present study, two-dimensional electrophoresis (DE) with immobilized pH gradients (IPG), matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) and bioinformatics were employed to detect the differentially expressed proteins between normal tissues and ESCC tissues. We identified 6 differentially expressed proteins among which the expressions and locations of HSP27 and ANX1 in the cancer tissues and normal tissues were further determined by immunohistochemistry.

Materials and Methods

Sample collection

The ESCC tissues and adjacent normal tissues were resected surgically from 50 patients with pathologically proven ESCC from August 2005 to September 2006. Tissues from 10 patients were used for proteomics – 6 males and 4 females with a mean age of 58 years (48–67 years). There were poorly differentiated squamous cell carcinomas (SCC) (n=4), intermediately differentiated SCC (n=5) and well-differentiated SCC (n=1). These tissues were washed with pre-cold PBS and stored in liquid nitrogen for use. The remaining tissues from 40 patients were fixed in 10% formaldehyde, embedded in paraffin and cut into sections (4 μm) followed by immunohistochemistry. These patients were 21 males and 19 females with a mean age of 59 years (40–75 years).

Reagents

IPG dry strip (pH3-10NL, 17 cm), IPG buffer (Bio-Lytes+ 3/10) (Bio-Rad, USA), urea, dithiothreitol (DTT), 3-(3-gall amide propyl)-diethyl ammonium)-propanesulfonic acid (CHAPS), Sodium Dodecylbenzenesulfonate (SDS), Tris base, acrylamide (Arc) methylene bisacrylamide (Bis), glycine, ammonium persulfate, iodine acetamide (IAA), mineral oil, glycerine, ammonium bicarbonate (NH4HCO3), TPCK-Trypsin, α-Cyano-3-hydroxycinnamic acid (CCA) (Sigma, USA), thiourea (Fluka, USA), formic acid (FA), acetonitrile (ACN) (Fisher, USA), trifluoroacetic acid (TFA) (Merck USA), sodium thiosulfate, sodium carbonate, silver nitrate, ethanol, glacial acetic acid, sodium acetate, paraffin, eosin, hematoxylin (analytically pure, China), concentrated goat anti-human Annexin I monoclonal antibody (ZYMED), concentrated mouse anti-human HSP27 monoclonal antibody (NeoMarker), EliVision™ plus IHC Ki and DAB (Fuzhou Maixin Biotech, China) were used in the present study.

Instruments

Isoelectric focusing apparatus (Protean IEF cell), vertical electrophoresis system (Protean II xi cell) (Bio-Rad, USA), transmission scanner (UMAX PowerLook 1100), image analysis software (PDQuest 7.1.0), Voyager DE STR MALDI-TOF (ABI, USA), ultraviolet spectrophotometer (DU-530; Beckman, USA) and microtome (Leica, Germany) were used in this study.

Protein extraction

Cancer tissues or normal tissues (0.9 g) were washed with PBS and ground in liquid nitrogen containing protease inhibitors (40 μg/mL PMSF, 1 mmol/L EDTA, 0.7 μg/mL pepstatin, 0.5 μg/mL leupeptin). These tissues were then added to 9 mL of lysis buffer (20 mmol/L Tirs, 7 mol/L urea, 2 mol/L thiourea, 40 μg/mL CHAPS, 2 mmol/L TBP, 0.2% IEF buffer) followed by ultrasonic processing (< 300 W) on ice. After this, centrifugation was performed at 105 000 g for 1 h at < 10 °C and the supernatant was collected and stored at –80 °C.

Determination of protein concentration

The protein concentration was determined by a modified Bradford method (5). BSA (1 mg/mL) and the Bradford working solution were prepared. Then 3.5 mL of the working solution were added to each tube followed by incubation for 5 min. The optical density was detected at 595 nm. The standard curve was delineated with Origin 7.0. The protein samples were serially diluted and the concentration of protein was detected and calculated according to standard curve.

Two-dimensional electrophoresis with immobilized pH gradients

The protein mixtures from cancer tissues and normal tissues were subjected to two-dimensional gel electrophoresis (2-DE). The 2-DE was carried out according to the manufacturer's instruction and method previously reported (6). In brief, 100 μg of proteins were mixed in loading buffer (7 mmol/L urea, 2 mmol/L thiourea, 4% CHAPS, 65 mmol/L DTT, 0.2% IEF buffer, trace amount of bromophenol blue) (total volume of 350 μL). Hydration of dry IPG strip was done at 20 °C for 16 h. Isoelectric focusing

(IEF) electrophoresis was conducted at a maximal current of 50 μA/gel at 20 °C. After a two-step process for equilibrium, the strips were transferred onto a separating gel (0.75 mm in thickness) followed by SDS-PAGE. Electrophoresis was performed first at a low current (12 mA/gel/17 cm) and then at a relatively high current (24 mA/gel/17 cm) when the samples were outside the IPG strips. The electrophoresis was discontinued when the index strip reached the bottom of the gel.

Silver staining

Silver staining of analytical gels was performed with silver nitrate as previously reported (7). The procedures included fixation, sensitization, washing, silver staining, washing, color development, reaction termination and washing. Blue silver staining for preparative gels was done as previously described (8).

Gel imaging and analysis

Transmission scanner (UMAX PowerLook 1100) was used to acquire the images which were then analyzed with PDQuest 7.1.0 software. The detection, quantitation, background removal and matching of spots were done. The spots with more than a 2-fold change were used for identification by MS.

Sample preparation for mass spectrometry

Protein spots were cut out from the gel and cut into small pieces (1–2 mm^2). After washing with water thrice, gel pieces were destained in 500 mL/L ACN and 25 mmol/L NH_4HCO_3 solution under continuous shake. The gel pieces were dehydrated in 100% CAN and then dried in a SpeedVac Evaporator for 50 min. The dried gel pieces were treated with 3–10 μL of 20 ng/mL trypsin solution supplemented with 25 mmol/L NH_4HCO_3 solution at 37 °C for 16–18 h. The peptide fragments were extracted in 5% trifluoracetic acid (TFA) for 1 h, then in 2.5% TFA/50% ACN for 1 h. Both samples were mixed and dried in an evaporator to 3–5 μL.

Mass spectrometry of proteins

In brief, 1 μL of extract liquor and 1 μL of matrix (α-Cyano-β-phenylacrylic acid in 1 mL/L TFA and 500 mL/L ACN) were injected into the column of a MALDI-TOF mass spectrometer (Voyager DE STR; ABI). The peptide mass spectra were obtained with the following settings: reflector positive ion mode, 20 kV accelerating voltage. Laser shots of 250 per spectrum were used to acquire the spectra with a minimal mass of 500 Da. Based on the signal to noise ratio, the spectrum of each sample was obtained from the cumulative spectra after laser bombardment of 50–200 times. Mass calibration was performed by using autolytic fragment peaks of trypsin including 2163.057 Da and 2273.160 Da. Proteins were identified by peptide fingerprinting using MS-Fit to search the NCBInr protein database (http://www.matrixscience.com). The criteria for database matching are 250 ppm mass tolerance, 1 fragment of incomplete digestion, cysteine carboaminomethylation as an intrinsic modification of proteins, methionine oxidation as selective modification, monoisotope, difference in the mass of peptide fragment of 0.01–0.1% and at least four peptides matched. The species of origin was restricted to Homo sapiens. MASCOT score of > 63 was considered statistically significant.

Immunohistochemistry

Immunohistochemistry was performed according to the manufacturer's instructions. The tissues were embedded in paraffin and sectioned followed by de-paraffinization, hydration and antigen retrieval. These sections were then incubated in 3% H_2O_2 solution for 10 min at room temperature to inactivate the endogenous hydrogen peroxidase and subsequently washed with PBS. These sections were treated with Annexin I antibody (1:200) or HSP27 antibody (1:500) at 4 °C overnight. After washing in PBS, sections were incubated with polymer enhancer at room temperature for 20 min. After another washing in PBS, sections were treated with the secondary antibody at room temperature for 30 min and again washed in PBS, followed by color development with DAB and counterstaining with hematoxylin. These sections were treated with 0.1% hydrochloric acid and then PBS followed by dehydration, transparentization and mounting. In the negative controls, the primary antibody was replaced with PBS. The staining intensity in immunohistochemistry was scored as follows: 0: no color; 1: light yellow; 2: brownish yellow; 3: brown. Based on the proportion of positive cells, sections were scored as follows: 1: 1–10%; 2: 11–50%; 3: 51–75%; 4: >75%. The product of both scores was the final score: 3: +; 4: ++; 5: +++ (+–+++ represent positive expression).

Statistical analysis

Statistical analysis was performed with the SPSS version 10.0 statistic software package and paired Wilcoxon signed ranks test was employed for comparisons. A value of $P<0.05$ was considered statistically significant.

Results

Protein expression profiling and protein identification by MS in ESCC

The proteins from ESCC tissues and normal tissues were subjected to 2-DE and the results were analyzed with PDQuest software. Results showed the

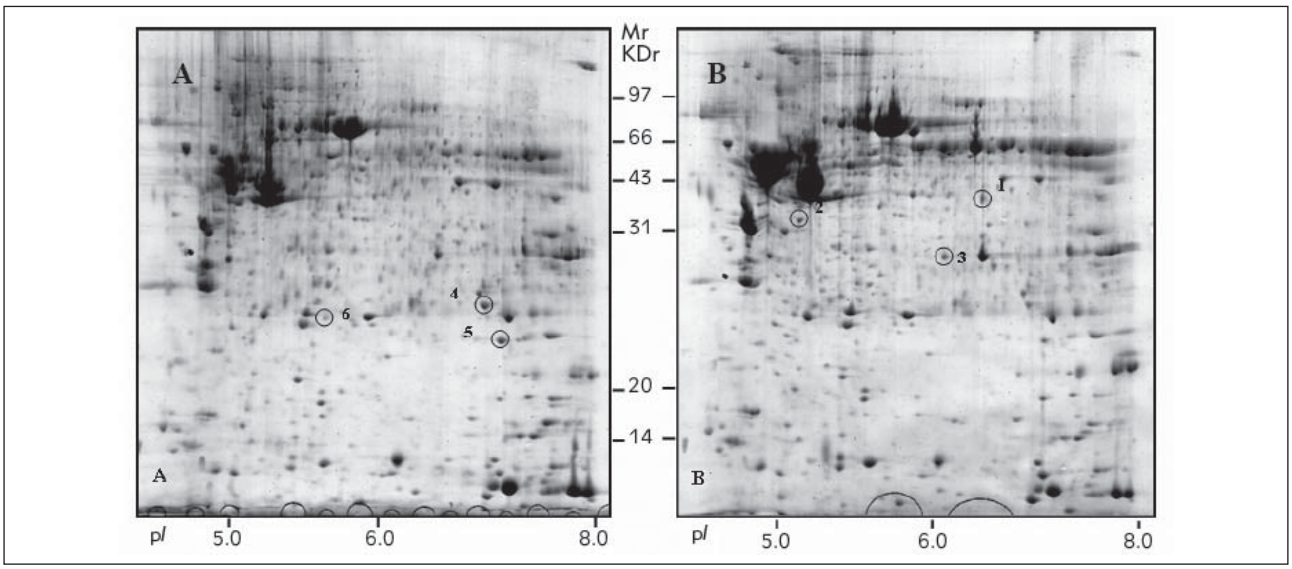

Figure 1 Protein expression profiling in ESCC and adjacent normal tissues. A: ESCC; B: adjacent normal tissues

Table I Differentially expressed proteins indentified by MALDI-TOF-MS.

Protein spot	MW/pI	Score	Coverage	Expression[a]	Database accession No.	Protein identification
6504	38552/6.28	75	22%	↓	Q9BYF8_HUMAN	(squamous cell carcinoma antigen 1)SCCA1b
2402	35750/5.1	192	65%	↓	Q9BTL1_HUMAN	KRT4 protein (Fragment)
6401	38787/6.64	190	57%	↓	ANXA1_HUMAN	Annexin A1 (Annexin I)
7206	26807/6.51	264	89%	↑	TPIS_HUMAN	Triosephosphate isomerase
7209	22290/6.86	81	69%	↑	1QNMA	manganese superoxide dismutase
4202	22427/7.83	78	50%	↑	E980237	HSAPIENS HSP27 SEQUENCE

a ↑: increased expression in ESCC; ↓: decreased expression in ESCC

pattern of the majority of proteins was similar between tissues. There were 48 spots showing significant difference between cancer tissues and normal tissues: 20 spots increased and the remaining 28 decreased in cancer tissues. Qualitative analysis (10-fold change in expression) showed 14 spots were found only in ESCC and 18 spots only in normal tissues. After analysis with software and manually screening, 6 differentially expressed proteins were selected and subjected to identification (*Figure 1*). After identification by MALDI-TOF-MS, the highly expressed proteins in ESCC included Triosephosphate isomerase 1 (TIM1), manganese superoxide dismutase (MnSOD) and Heat shock protein 27 (HSP27) and those in normal tissues consisted of squamous cell carcinoma antigen 1 (SCCA1), keratin 4, type II, cytoskeletal (KRT4) and Annexin A1 (ANX1) (*Table I*).

Immunohistochemistry for HSP27 and ANX1 in the ESCC and normal tissues

Immunohistochemistry was performed in 40 ESCC tissues and corresponding normal tissues. Results showed HSP27 was mainly found in the cytoplasm and was brownish yellow in ESCC but colorless or light yellow in normal tissues (*Figure 2*). The expression of HSP27 in the cancer tissues was markedly high-

Table II Scores of HSP27 expression in ESCC and normal tissue.

Group		Adjacent normal tissue			
		–	+	++	+++
ESCC	+	0	4	0	0
	++	3	12	2	0
	+++	7	9	2	1

Z=5.094, P<0.001

Table III Scores of Annexin I expression in ESCC and normal tissue.

Group		Adjacent normal tissue		
		–	+	++
ESCC	–	4	6	2
	+	0	19	4
	++	1	2	1
	+++	0	1	0

Z=5.094, P<0.001

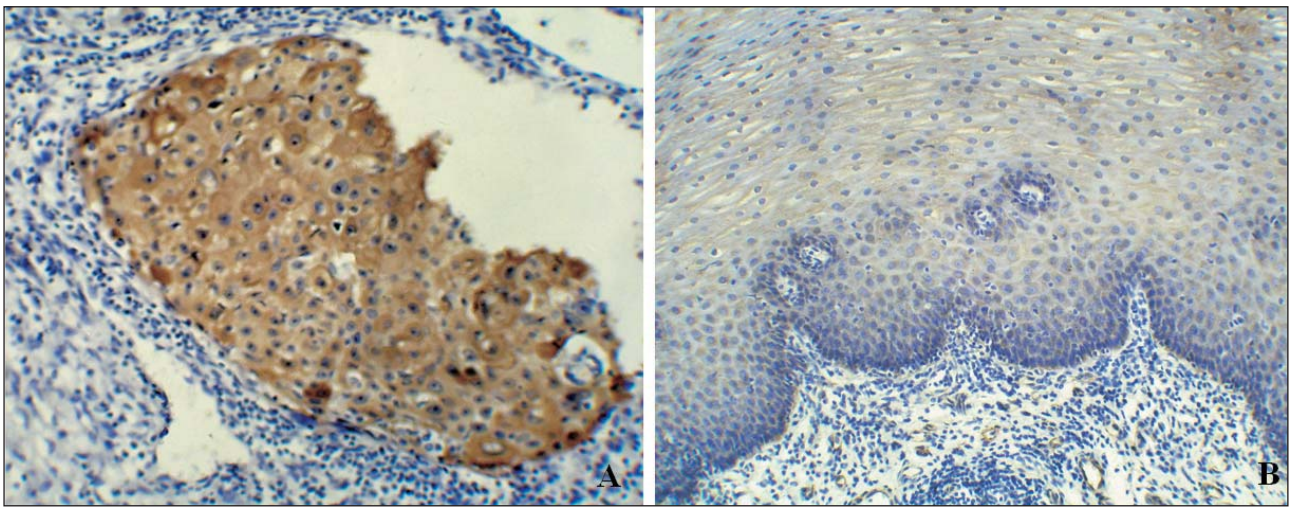

Figure 2 HSP27 expression in the ESCC and adjacent normal tissues (200×) A: ESCC; B: Corresponding normal tissue

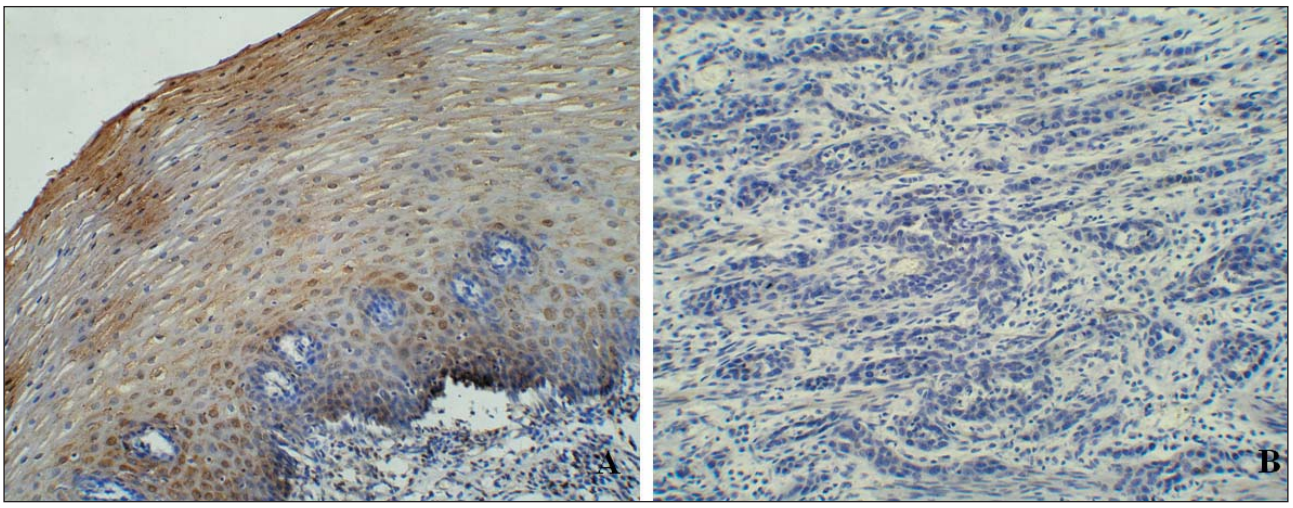

Figure 3 3 Annexin I expression in the ESCC and adjacent normal tissues (200×) A: ESCC tissue; B: Corresponding normal tissue

er than that in normal tissues (Z=5.094, P<0.001) (*Table II*). In addition, ANX1 was also found in the cytoplasm and was light yellow or brownish yellow in the normal tissues, but light yellow or colorless in ESCC, although brown staining was found in several cancer cells (*Figure 3*). Analysis showed the ANX1 expression was significantly lower in ESCC than in normal tissues (Z=5.197, P<0.001) (*Table III*).

Discussion

Cancer cells were transformed from normal cells. Thus, these cells not only have the specific proteins that normal cells haven't, or don't express some specific proteins that can be found in normal cells, but have a large amount of proteins similar to normal cells. However, the modification and/or processing of these similar proteins in cancer cells are abnormal at the transcriptional and/or translational level. Through comparing the proteome among different patients with the same cancer or between cancer cells and their original normal cells, some cancer-specific proteins may be identified. The in-depth studies on the structure and functions of these specific proteins may be helpful for the understanding of the pathogenesis of cancers, for the diagnosis and treatment of cancers and for the development of new anti-tumor drugs.

In the present study, we compared the proteomes in human ESCC and adjacent normal tissues, and a total of 6 differentially expressed proteins were identified, among which SCCA1, KRT4 and ANX were markedly down-regulated in ESCC, and TIM1, MnSOD and HSP27 significantly up-regulated. For protein spots in 2-DE with significant fold change, MALDI-TOF-MS can be used as a sensitive tool to identify these proteins. To further confirm our findings in proteomics, 40 patients with ESCC were recruited and immunohistochemistry for ANX1 and HSP27 was performed in ESCC tissues and adjacent normal tissues of these patients. Our results showed the HSP27 was highly expressed in ESCC, which however had a low expression of ANX1, consistent with the findings in proteomics.

HSP has a low molecular weight and is widely distributed in the normal cells of various species and cancer cells. The HSP27 mainly acts as a molecular chaperone to protect the cells against injury by stress. Studies have demonstrated there is difference in the HSP27 expression between cancers and normal tissues, and the level and functions of HSP27 vary by cancer type. Leonardi et al. (9) found that the HSP27 expression in normal oral mucosa was higher than that in precancerous lesions and squamous cell carcinoma, and down-regulation of HSP27 was found in poorly differentiated cancers and up-regulation in well-differentiated cancers. However, in gastric cancer and breast cancer, HSP27 expression was significantly higher than in normal tissues, and high expression of HSP27 predicted a poor prognosis. The biological role of HSP27 in cancers may be related to the promotion of cell proliferation and suppression of apoptosis of cancer cells by HSP27 (10, 11). Our study showed the HSP27 expression in ESCC was dramatically higher than that in corresponding normal tissues, suggesting HSP27 plays a role in the pathogenesis of ESCC.

ANX1 is one of the members of the annexin family. The annexins are a structurally conserved family of proteins characterized by reversible Ca^{2+}-dependent phospholipid binding. ANX1 can bind to not only the Ca^{2+} and phospholipids, but the actin involved in some cellular processes including intracellular signal transduction, cell differentiation and apoptosis. ANX1 exerts different effects in different cancers. Studies have indicated ANX1 was associated with the occurrence of prostate cancer (12) and pancreatic cancer (13). Paweletz et al. (14) found that the loss or decreased expression of ANX1 was related to the early events in esophageal cancer. In the present study, our results showed ANX1 expression in ESCC was significantly lower than in normal tissues, which was consistent with the study of Du et al. (15). These results suggest the occurrence of ESCC may be associated with the loss of ANX1, and the ANX1 gene may act as a tumour suppressor gene in ESCC.

SODs are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen. Yokoe et al. (16) reported that MnSOD was highly expressed at the transcriptional level in oral SCC tissue, compared with normal tissue counterparts, whereas no significant changes in Cu/ZnSOD were detected. Up-regulation of MnSOD has been reported in ESCC (15, 17). A paradigm for SOD function in cancer is based on the potency of a cellular defense mechanism against ROS. Qi et al. also speculated the up-regulation of MnSOD in ESCC might reflect the cell defense effort in maintaining intracellular homeostasis (17).

Squamous cell carcinoma antigen (SCCA) is a sub-fraction of TA-4, a tumour-associated antigen first described by Kato et al. in 1977 (18), and belongs to the family of serine protease inhibitors (serpins) (19). Serpins were found to be involved in a variety of biological functions, including fibrinolysis, coagulation, inflammation, tumour cell invasion, cellular differentiation, and apoptosis (20). The SCCA was found highly up-regulated in various SCC cancers including those in the uterine cervix, lung, head and neck, skin (21) and recently in buccal mucosa (18); this protein was therefore regarded as an SCC tumor marker. However, our results showed the SCCA1 was down-regulated in ESCC. This finding, although contradictory to the results from previous studies with other SCC cancers, implicates that the SCCA1 may have a unique function in esophagus SCC tumorigenesis. Therefore, it is likely that SCCA1 is an indicator for the histological differentiation of the ESCC (22).

KRTs are a family of about 30 different related cytoplasmic proteins encoded by multiple genes and constitute the intermediate filaments that are incorporated into the cytoskeleton of almost all epithelial cells (23). KRTs are the major constituents of the normal esophageal epithelium and epithelial cancer (24). In the past, several reports have shown alterations in the CK protein expression in the normal

esophageal epithelium in comparison to ESCC. Chung et al. (25) demonstrated the reduction of CK4 and CK14 in the transition from normal epithelium to invasive tumour in a small number of cases. Xue et al. (26) studied 205 ESCC and 173 precursor lesions and found that the underexpression of CK4 in the ESCC was an early event occurring in the mild to moderate dysplasia stage and suggesting malignant transformation in this cancer. The down-regulation of CK4 was also confirmed in the study of Singh et al. (27) by immunohistochemistry. These findings were consistent with ours.

TIM is an enzyme that catalyzes the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. It is ubiquitously distributed in the cytoplasm of all tissues, and generally high levels are found in tissues with high glycolytic activity. Previous reports have shown its overexpression in lung adenocarcinoma and squamous cell carcinoma of the bladder (28, 29). In addition, over-expression of TIM may be a cause of multidrug resistance of gastric cancer (30). However, few studies were performed to investigate the role of TIM in esophageal cancer. More studies are needed to clarify the biological role of TIM1 in the pathogenesis of ESCC. Taken together, our study showed there was difference in the protein expression profiling between ESCC and adjacent normal tissues. We employed proteomics and immunohistochemistry to determine the differentially expressed proteins between ESCC and adjacent tissues. Our results provide evidence for the studies on the molecular pathogens of ESCC. More studies are required to investigate the roles of differentially expressed proteins in the occurrence and development of cancers.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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