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ORIGINAL ARTICLE

Quantitative Proteomics Identify the Possible Tumor Suppressive Role of Protease-Activated Receptor-4 in Esophageal Squamous Cell Carcinoma Cells

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Abstract
Exposure to carcinogens of tobacco smoke may result in methylation of protease-activated receptors-4 (PAR4) gene and further induces the loss of PAR4 expression, which is considered to be involved in carcinogenesis of esophageal squamous cell carcinoma (ESCC). Here we employed a TMT-based quantitative proteomic approach to identify PAR4-regulated changes of proteomic profiles in ESCC cells and to identify potentially therapeutic value. A total of 33 proteins were found significantly changed with 15 up-regulated and 18 down-regulated in PAR4-activating peptide (PAR4-AP) treated ESCC cells compared with controls. Bioinformatics analysis showed that key higher expressed proteins included those associated with apoptosis and tumor suppressor (e.g. CASP9), and lower expressed proteins included those associated with anti-apoptosis, autophagy and promoting cell proliferation (e.g. CHMP1B, PURA, PARG and HIST1H2AH). Western blot verified changes in five representative proteins including CASP9, CHMP1B, PURA, PARG and HIST1H2AH. Immunohistochemistry analysis showed that CHMP1B, PURA, PARG and HIST1H2AH expression in ESCC tissues were significantly higher than those in adjacent nontumorous tissues. Our findings will be helpful in further investigations into the functions and molecular mechanisms of PAR4 in ESCC.

Keywords Proteomic · Protease-activated receptors-4 · Apoptosis · Tumor suppressor · Esophageal squamous cell carcinoma

Introduction

Protease-activated receptors (PARs), a family of G-protein coupled receptors, are activated by proteases released by cell damage or blood clotting which lead to a specific cellular response [1]. PAR4, the fourth and last identified PAR member which is activated by thrombin, was reported to be related to the development of cancers [2].

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive types of malignancy with a poor prognosis in the world [3]. Tobacco smoking has long been recognized as an important cause of ESCC [4, 5]. Exposure to carcinogens of tobacco smoke may result in methylation of PAR4 gene (also known as coagulation factor II receptor-like 3, F2RL3) and further induce the loss of PAR4 expression, which is considered to be involved in carcinogenesis [6, 7]. PAR4 is highly expressed in human esophagus squamous epithelial cells [8], and frequently down-regulated in ESCC tissue which was partly resulted from the hypermethylation of the PAR4 promoter [7], but the role of PAR4 in the progress of ESCC is still unknown. Herein, we employed a tandem mass tag (TMT)-based quantitative proteomic approach to identify PAR4-regulated changes of proteomic profiles in ESCC cells, and to identify potentially diagnostic or therapeutic value.

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Materials and Methods

Cell Culture and Drug Administration

Human esophageal squamous cell carcinoma (ESCC) cell lines (EC9706 and EC109) were obtained from the Huiying Biological

Technology (China) or Baiou Bowei Biological Technology (China). Cell lines were cultured in RPMI-1640 medium (Gibico, USA) supplemented with 10% fetal bovine serum (FBS, Biological Industries) incubated at 37 °C in an atmosphere of 5% CO2. ESCC cells were continuously stimulated with AYPGKF-NH2 (selective PAR4-activating peptide, PAR4-AP, Bachem, Weil-am-Rhein, Germany) at a concentration of 100 μM for 6 h. Cells without PAR4-AP were used as control.

Protein Digestion and Peptide Tandem Mass Tag (TMT) Labeling

Protein digestion and TMT labeling were done as previously described [9]. TMT labeling was carried out according to the manufacturer instructions. Briefly, tryptic peptides from each sample was reconstituted in 100 μl of 50 mM Tris 2-carboxyethyl phosphine (TCEP) buffer and mixed with the TMT10plex Isobaric Label Reagent Set (Thermo Scientific, Rockford, USA) reconstituted in 41 μl of anhydrous acetonitrile (ACN), incubated at room temperature for 1 h. All the labeled peptides from each sample were equally mixed, dried completely in a vacuum concentrator and kept at −80 °C.

LC-MS/MS Analysis

Peptides were separated and analyzed with a Nano-HPLC (EASY-nLC1200, Thermo Scientific) coupled to Q-Exactive mass spectrometry (Thermo Finnigan). High mass resolution was utilized for peptide identification and highenergy collision dissociation (HCD) was used for reporter ion quantification as previously described [9].

Database Search and TMT Quantification

Raw MS files were processed with MaxQuant (Version 1.5.6.0). The HUMAN protein sequence database (Uniprot_HUMAN_2016_09) was downloaded from UNIPROT. This database and its reverse decoy were then searched against by MaxQuant software. The ratios of TMT reporter ion abundances in MS/MS spectra generated by HCD from raw data sets were used For TMT quantification. Fold changes in proteins between control and treatment were calculated as previously described [9, 10].

Bioinformatics Analysis

The Gene Ontology (GO) functional and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed for the differential expression of proteins using the Database for Annotation, Visualization and Integrated Discovery (DAVID) and KEGG Orthology-Based Annotation System (KOBAS) online tool. Hierarchical cluster analysis was performed for enriched proteins by Cluster 3.0 software.

Western Blot Analysis

ESCC cells were continuously stimulated with AYPGKF-NH2 at a concentration of 100 μM for 1, 2, 6, 12 and 24 h. Then, cells were lysed, and protein was extracted. Protein lysate from each sample was separated electrophoretically in sodium dodecyl sulfatepolyacrylamide gel, and then transferred to polyvinylidene fluoride (PVDF) membranes. Western blot analyses were performed with anti- CASP9 (CST, Danvers, MA), HIST1H2AH (GeneTex, Irvine, CA), CHMP1B (Sigma-Aldrich, St. Louis, MO), PURA (Novus Biologicals, Littleton, CO) and PURG (Abcam, Cambridge, UK).

Immunohistochemistry Analysis

Twenty-six ESCC tissues and their corresponding nearby non tumorous tissues were obtained from the Affiliated Hospital of Taishan Medical University, China, with the approval of the Local Research Ethics Committee. ESCC specimens were fixed in 10% buffered formalin. Paraffin sections were stained with anti- HIST1H2AH (GeneTex), CHMP1B (Sigma-Aldrich), PURA (Novus Biologicals) and PURG (Abcam), then counterstained with hematoxylin.

Immunoreaction scores were based on the percentage and intensity of positively stained cells. The percentage score was as follows: no positive cells (0), fewer than 25% positive tumor cells (1), 25% to 50% positive tumor cells (2), 51% to 75% positive tumor cells (3), and greater than 75% positive tumor cells (4). The intensity score was as follows: no staining (0), light yellow (1), yellow-brown (2), and dark brown (3). The immunoreaction score was then calculated by multiplying the percentage and intensity scores.

Statistical Analysis

Each experiment was repeated at least three times. Values are expressed as mean ± SEM and results were analyzed using an ANOVA followed by Bonferroni test for comparison between groups for immunoreactive levels in matched tumor tissues and adjacent nontumor tissues. Significance was defined as P-values <0.05.

Results

PAR4 Influenced Proteomic Enrichment Profiles in ESCC Cells

The differential expression of proteins between control and treated with PAR4-AP human ESCC cell lines was determined by tandem mass tag (TMT) labeling quantitative proteomic approach (Supplementary Fig. 1).

Using a fold change of at least 1.25 and *P*-values ≤0.01, enrichment profiles were compared to those of treatment with control. Over 5225 proteins were identified from control and PAR4-AP treated human ESCC cells. Among them, some 33 proteins were significantly changed upon PAR4-AP treatment, including 15 up-regulated, and 18 down-regulated proteins when compared to ESCC control cells (Fig. 1a; Table 1).

Bioinformatics Analysis of Proteomic Enrichments Induced by PAR4

GO analysis showed that ten GO terms were enriched for differential expression of proteins, which were mainly related to chaperone-mediated autophagy, extracellular exosome, poly(A) RNA binding, ATPase activity, cellular response to UV, cellular response to starvation, and DNA damage

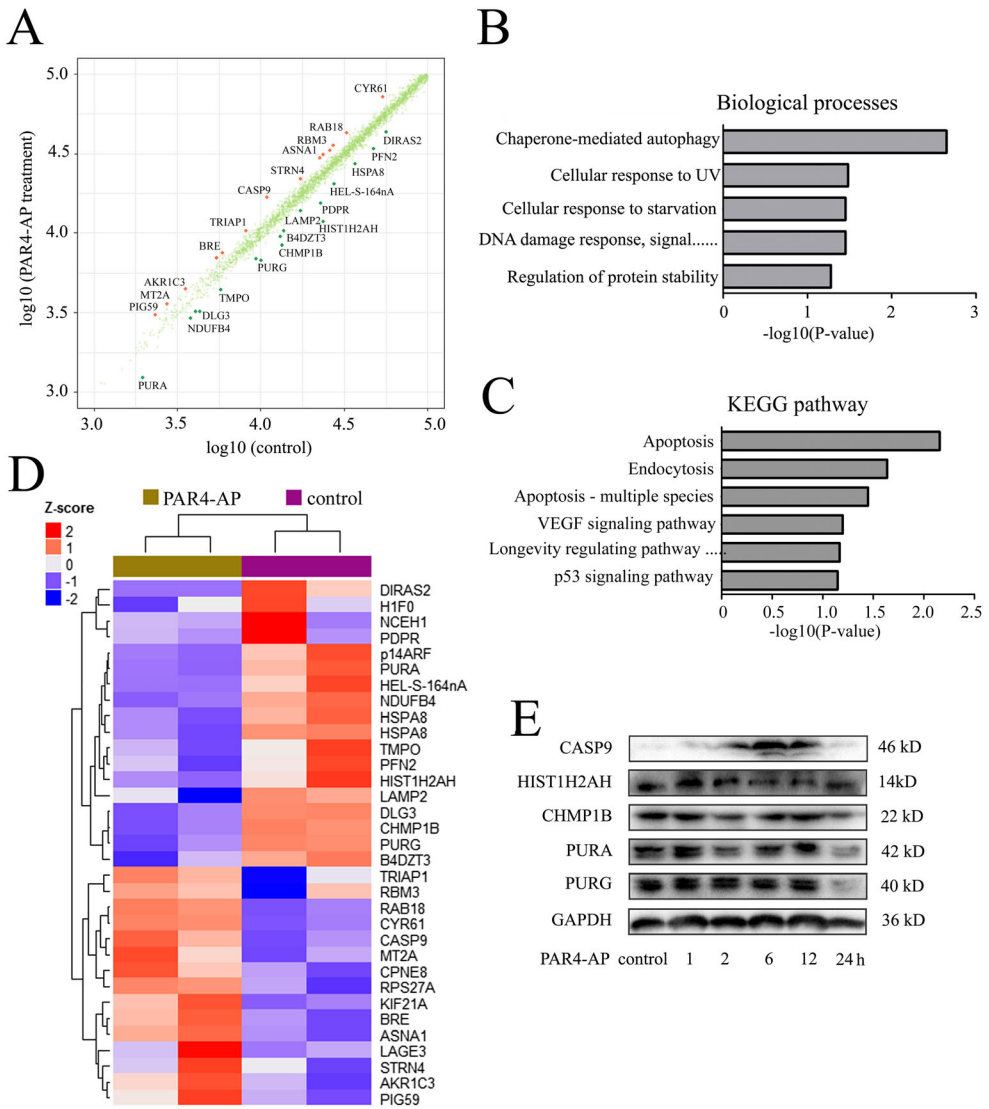

Fig. 1 The differential expression of proteins between control and PAR4-AP treated ESCC cells was determined by TMT approach. **a** Differentially expressed proteins in ESCC cells following treatment with PAR4-AP (100 μM) at 6 h, compared to control group. Blue dots represent proteins with significantly down-regulated expression, red dots represent proteins with significantly up-regulated expression, while pale green dots represent proteins with no significantly difference. **b** Gene Ontology analysis showing the significant GO terms (biological processes) of enriched proteins after treatment of PAR4-AP, compared with control group. **c** Statistically significant KEGG pathways of enriched proteins after treatment of PAR4-AP, compared with control group. **d** Hierarchical cluster analysis of the proteins with a significantly regulated expression profile between PAR4-AP treated ESCC cells and control group (R-package, ComplexHeatmap). **e** Representative western blots confirmed changes in protein expression initially identified by quantitative proteomics method. HIST1H2AH, CHMP1B, PURA and PURG were found down–regulated in PAR4-AP treated ESCC cells compared with controls. Up-regulated expression was observed for CASP9. GAPDH was used as the loading control

Table 1 The PAR4-regulated proteins in ESCC cells

Gene symbols	Description	Change fold	*P*-value	Biological process
CASP9	Caspase-9	1.53	0.0045	apoptotic process
CYR61	Cysteine rich angiogenic inducer 61	1.33	0	regulation of cell growth
RAB18	Ras-related protein Rab-18	1.31	0	small GTPase mediated signal transduction
PIG59	Cell proliferation-inducing protein 59	1.31	0	glutamate metabolic process
RBM3	RNA-binding protein 3	1.30	0.0002	RNA processing
MT2A	Metallothionein-2	1.30	0.0004	cellular response to drug
ASNA1	ATPase ASNA1	1.30	0	IRE1-mediated unfolded protein response
RPS27A	Ribosomal protein S27a	1.30	0	G2/M transition of mitotic cell cycle
CPNE8	Copine-8	1.28	0.0002	
KIF21A	Kinesin-like protein KIF21A	1.28	0	microtubule-based movement
TRIAP1	TP53-regulated inhibitor of apoptosis 1	1.27	0.0008	apoptotic process
BRE	BRCA1-A complex subunit BRE	1.27	0	apoptotic process
LAGE3	EKC/KEOPS complex subunit LAGE3	1.26	0.0002	tRNA processing
STRN4	Striatin-4	1.26	0.0020	
AKR1C3	Aldo-keto reductase family 1 member C3	1.25	0	G-protein coupled receptor signaling pathway
H1F0	Histone H1.0	−1.25	0	apoptotic DNA fragmentation
LAMP2	Lysosome-associated membrane glycoprotein 2	−1.26	0.0069	protein stabilization
p14ARF	p14ARF/p16INK4a fusion protein	−1.27	0.0020	G1/S transition of mitotic cell cycle
DIRAS2	GTP-binding protein Di-Ras2	−1.30	0	small GTPase mediated signal transduction
NDUFB4	NADH dehydrogenase 1b subcomplex subunit 4	−1.31	0	response to oxidative stress
TMPO	Thymopoietin	−1.32	0	regulation of transcription, DNA-templated
HEL-S-164 nA	Neutral alpha-glucosidase AB, GANAB	−1.34	0	carbohydrate metabolic process
DLG3	Disks large homolog 3	−1.34	0	negative regulation of cell proliferation
B4DZT3	cDNA FLJ50934, highly similar to Lamin-B1	−1.34	0.0014	
NCEH1	Neutral cholesterol ester hydrolase 1	−1.36	0	metabolic process
HSPA8(E9PN89)	Heat shock protein family A (Hsp70) member 8	−1.37	0	chaperone-mediated autophagy
HSPA8(Q96IS6)	HSPA8 protein	−1.39	0	cellular response to starvation
PFN2	Profilin-2	−1.39	0	actin cytoskeleton organization
PDPR	Pyruvate dehydrogenase phosphatase regulatory subunit	−1.48	0	oxidation-reduction process
PURG	Purine-rich element binding protein gamma	−1.50	0	
PURA	Purine rich element binding protein alpha	−1.60	0.0004	DNA replication initiation
CHMP1B	Charged multivesicular body protein 1b	−1.61	0.0062	cell cycle
HIST1H2AH	Histone cluster 1 H2A family member h	−2.02	0	chromatin silencing

response, signal transduction by p53 class mediator resulting in cell cycle arrest (Fig. 1b). Based on KEGG analysis, apoptosis and p53 signaling pathway were relevant to PAR4-AP treatment (Fig. 1c). Therefore, alterations of these proteins mainly regulated by PAR4 could have severe consequences in ESCC cell biology, such as apoptosis, autophagy and cell cycle arrest.

Hierarchical Cluster Analysis of Proteomic Enrichments Induced by PAR4

Enriched proteins were hierarchically clustered dependent on the protein enrichment features of control against PAR4-AP treated cells (Fig. 1d). Key higher expressed proteins induced by PAR4-AP in ESCC cells included those associated with apoptosis and tumor suppressor, such as caspase-9 (CASP9) and BRCA1-A complex subunit BRE (BRE) [11, 12]. Furthermore, higher expression of proteins in ESCC control cells included those associated with anti-apoptosis, cell growth, autophagy and chromatin silencing, such as heat shock protein family A (Hsp70) member 8 (HSPA8) [13], lysisome-associated membrane glycoprotein 2 (LAMP2) [13], purine-rich element binding protein A (PURA) [14], charged multivesicular body protein 1b (CHMP1B) [15], and histone cluster 1 H2A family member h (HIST1H2AH) [16]. Most of these proteins have been previously reported to regulate apoptotic

process in cancers and induce apoptosis via mitochondrial cell death pathway (e.g. CASP9) [11]. This clustering analysis of TMT data indicates that the PAR4-induced protein alteration is closely associated with the apoptotic process, autophagy and chromatin silencing of ESCC cells.

Validation by Western Blot

Western blot analyses were performed on selected candidates (CASP9, HIST1H2AH, CHMP1B, PURA and PURG). These candidates were chosen based on proteins with at least 1.5-fold change after 6 h incubation

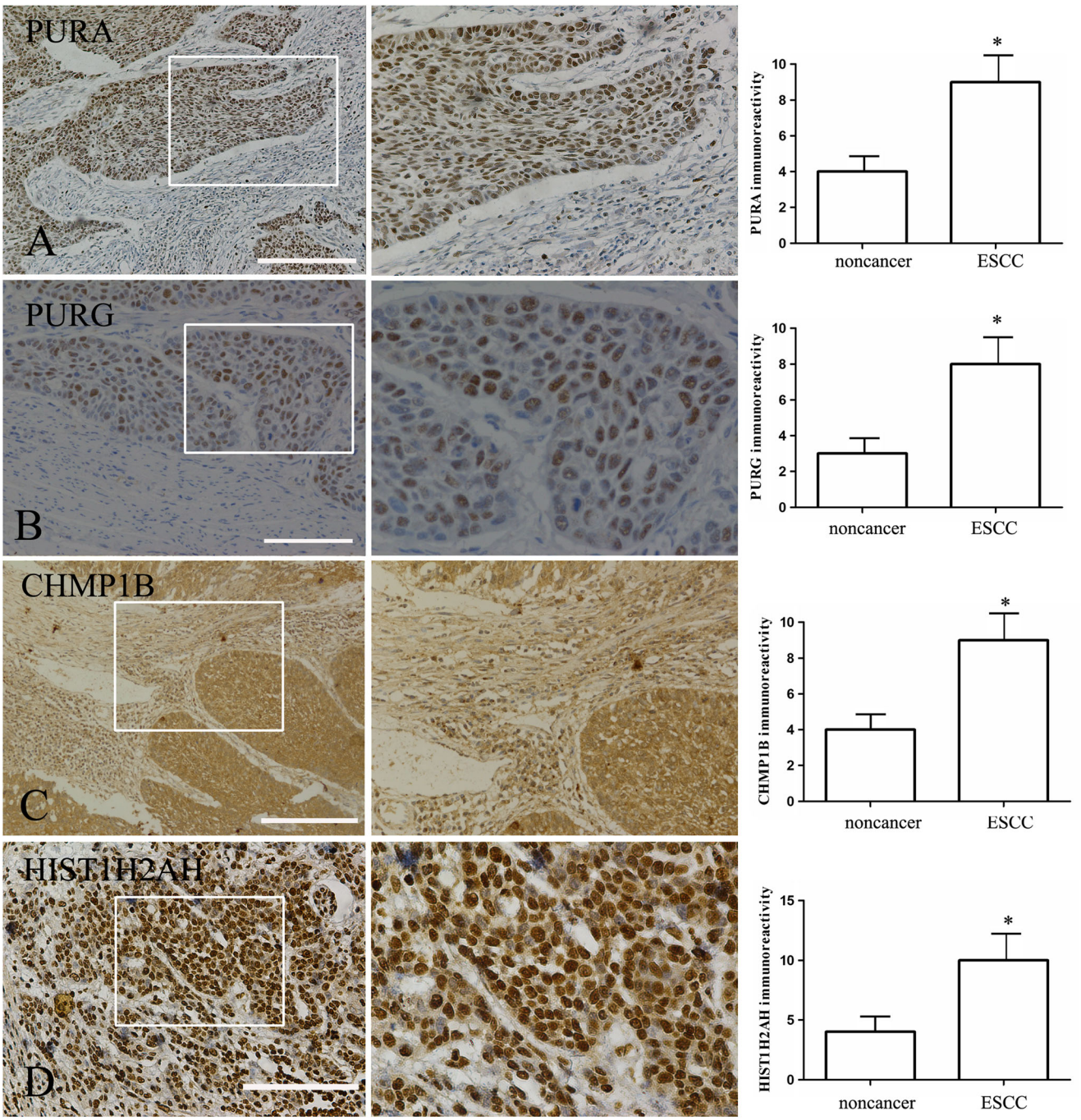

Fig. 2 Immunohistochemical staining for PURA, PURG, CHMP1B and HIST1H2AH in ESCC tissues. Representative immunostaining for PURA (**a**), PURG (**b**), CHMP1B (**c**) and HIST1H2AH (**d**) are shown. Sections were counterstained with hematoxylin. Scale bars 100 μm (**a**, **c**), 50 μm (**b**, **d**). Graphs showing PURA, PURG, CHMP1B and HIST1H2AH immunostaining in ESCC and adjacent noncancerous tissues ($n = 26$). The data are expressed as the mean ± SEM. *$p < 0.01$ (Student's t-test). Immunoreactivity was calculated by multiplying the percentage and intensity scores

of ESCC cells with PAR4-AP. Figure 1e shows that the up- or down-regulation trend of candidate proteins between PAR4-AP treatment and control group revealed by the Western blot data is congruent with that revealed by quantitative proteomic method. The result of Western blotting provides evidence that the TMT labeling method for the large scale protein quantification was reliable.

Expression of CHMP1B, HIST1H2AH, PURA and PURG in ESCC Tissues

Next, we assessed the CHMP1B, HIST1H2AH, PURA and PURG expression in 26 paired ESCC and adjacent nontumorous tissues by immunohistochemistry. HIST1H2AH, PURA and PURG were homogenously expressed and manifested as nuclear staining in ESCC cells, and CHMP1B immunoreactivity was observed in ESCC tissues with positive signal in cytoplasm and perinuclei of neoplastic cells (Fig. 2). Immunohistochemistry analysis showed that the expression levels of all four proteins were significantly higher in ESCC than those in adjacent nontumor tissues (Fig. 2b; $p < 0.01$). CHMP1B, HIST1H2AH and PURG have not been previously reported in ESCC, and PURA has previously been linked to this cancer.

Discussion

In the present study, we used a TMT-based quantitative proteomic approach, which has been applied to a wide range of cancer studies [9, 10], and examined the influence of PAR4 on the regulation of proteomic profiles to test the hypothesis that PAR4 was associated with a tumor suppressor role ESCC. Bioinformatics analysis showed that differentially expressed proteins were associated with chaperone-mediated autophagy, apoptosis and p53 signaling pathway. The proteomic results were consistent with the Western blotting and immunohistochemistry analysis analyses. Our study presents evidence at a proteomic level that PAR4 represents a novel candidate therapeutic target in ESCC cells.

Accumulating evidence demonstrates that PAR4 is significantly down-expressed in gastric carcinoma and lung adenocarcinoma, and that selective loss of PAR4 expression might result from hypermethylation of the PAR4 promoter [6, 17]. PAR4 is highly expressed in human esophagus squamous epithelial cells, and mainly down-regulated in ESCC tissue which was partly resulted from the increased methylation level of PAR4 promoter [7]. In the present study, we found that activation of PAR4 could alter protein expression profiles in ESCC cells. In order to obtain insights into PAR4 target gene function, GO and KEGG analysis annotation were applied. Bioinformatics analysis showed that key higher expressed proteins included those associated with apoptosis and tumor suppressor, and lower expressed proteins included those associated with anti-apoptosis, autophagy and promoting cell proliferation. Therefore, alterations of these proteins mainly regulated by PAR4 could have severe tumor suppressive role in ESCC cell biology.

Among identified candidate proteins, CASP9 has an important role in the mitochondrial cell death pathway as the initiator of apoptosis [11]. HIST1H2AH, PURA, PURG and CHMP1B might be associated with the silencing of tumor suppressor genes, autophagy, anti-apoptosis, and accelerated cell proliferation [12–16]. CHMP1B, HIST1H2AH and PURG have not been previously reported in ESCC. Ubiquitination of histone H2A might be involved in the silencing of tumor suppressor genes and promote breast cancer growth [16, 18]. PURA plays an important role in inhibition of CASP8 activity and anti-apoptosis in ESCC cells [14]. Quantitative RT-PCR analysis showed that PURG levels are low or undetectable in normal tissues, but they are greatly elevated in tumor tissues [19]. Charged multivesicular body proteins are involved in autophagy, anti-apoptosis, and accelerated cell proliferation [15, 20, 21]. Taken together with our results, this indicates that the up-regulation of CASP9 and down-regulation of HIST1H2AH, PURα, PURG and CHMP1B by PAR4 may contribute to cell growth inhibition and apoptosis in ESCC cells. Previous studies demonstrated that mice with PAR4 knockout accelerated tumor growth and reduced cardiomyocyte apoptosis [22, 23], similar to what we observed in ESCC cells induced by PAR4-AP. Our findings might provide some evidence regarding its potential tumor suppressive role through apoptosis and inhibiting ESCC cell growth. These findings suggest that PAR4 may be a potential new target for the treatment of ESCC.

In conclusion, a proteomic approach was used to investigate the tumor suppressive effect of PAR4 ESCC cells. Five key proteins were identified; however, further studies are needed to confirm our results. Our proteomic study provides a global characterization of protein expression of PAR4-AP-treated human ESCC cells.

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Compliance with Ethical Standards

Conflict of Interest The authors have declared that no competing interest exists.

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