Protease-activated receptor (PAR)1, PAR2 and PAR4 expressions in esophageal squamous cell carcinoma

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Abstract: Here, we used reverse transcription-PCR (RT-PCR) and western blot to detect protease-activated receptor (PAR) 1, PAR 2 and PAR 4 expression in cancer tissues and cell lines of esophageal squamous cell carcinoma, and investigated the co-relationship between PAR expression and clinic-pathological data for esophageal cancer. The methylation of *PAR4* gene promoter involved in esophageal carcinoma was also analyzed. By comparing the mRNA expressions of normal esophageal tissue and human esophageal epithelial cells (HEEpiC), we found that among the 28 cases of esophageal squamous cell carcinoma, *PAR1* (60%) and *PAR2* (71%) were elevated in 17 and 20 cases, respectively, and *PAR4* (68%) expression was lowered in 19 cases. Whereas, in human esophageal squamous cells (TE-1 and TE-10), *PAR1* and *PAR2* expression was increased but *PAR4* was decreased. Combined with clinical data, the expression of PAR1 in poorly differentiated (P=0.016) and middle and lower parts of the esophagus (P=0.016) was higher; expression of PAR4 in poorly differentiated carcinoma was lower (P=0.049). Regarding TE-1 and TE-10 protein expression, we found that in randomized esophageal carcinoma, PAR1 (P=0.027) and PAR2 (P=0.039) expressions were increased, but lowered for PAR4 (P=0.0001). In HEEpiC, TE-1, TE-10, esophageal and normal esophagus tissue samples (case No. 7), the frequency of methylation at the 19 CpG loci of *PAR4* was 35.4%, 95.2%, 83.8%, 62.6% and 48.2%, respectively. Our results indicate that the expression of PAR1 and PAR2 in esophageal squamous cell carcinoma is increased but PAR4 is decreased. Hypermethylation of the promoter of the *PAR4* gene may contribute to reduced expression of PAR4 in esophageal squamous cell carcinoma.

Keywords: PAR1; PAR2; PAR4; Esophageal squamous cell carcinoma; PCR; Western Blot; Methylation

The development of esophageal squamous cell carcinoma (ESCC) is a complicated process with multiple pathological stages. Among various regulatory factors, proteases play critical roles in activating signal transduction pathways and regulating gene expression (Ikeda et al, 1999). Protease-activated receptors (PARs) are a subfamily of the single seven-transmembrane G-protein-coupled receptors and include PAR1, PAR2, PAR3 and PAR4 (Macfarlane et al, 2001). PAR1, PAR3 and PAR4 are thrombin receptors, and PAR2 is trypsinase/tryptase receptor (Xu et al, 1998). Ribeiro et al (2009) found high expression of PAR1 but low expression of PAR2 in the tissues of ESCC; however, Wang et al (2010) found high expression of PAR2 in ESCC. PAR4 is a recently discovered novel subtype expressed in high amounts in colon cancer and hepatocarcinoma and induces the proliferation and migration of cancer cells (Gratio et al, 2009; Kaufmann et al, 2007). PAR4 expression in stomach cancer tissues (Zhang et al, 2011) and adenocarcinoma of the lung are low (Jiang et al, 2013), but its expression in ESCC remains unclear.

Here, we determined the expression of PAR1, PAR2 and PAR4 in tissues of ESCC and analyzed correlations

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between *PAR4* promoter hypermethylation and the development of ESCC. Our aim was to provide theoretical evidence for clinical diagnosis, treatment and prognosis of ESCC and provide a basis for drug research.

MATERIALS AND METHODS

Experimental materials

Human esophageal epithelial cells (HEEpiC) and human esophageal squamous cells (TE-1, TE-10) were obtained from the Cell Bank of Kunming Institute of Zoology, Chinese Academy of Sciences. Tissues of 28 cases (male=21, female=5; 51-81 years old) of diagnosed ESCC were from hospitals affiliated with the Kunming Medical University, Yunnan, China. All patients were clean of any chemotherapy or radiation treatment prior to surgery. The carcinoma tissues and corresponding normal control tissues (at least 5 cm away from carcinoma tissue) obtained during surgery were quick frozen in liquid nitrogen and stored at –80 °C. All the experimental protocols were approved by the Ethics Committee of Kunming Medical University.

Experimental procedures Cell culture

TE-1 and TE-10 cells were cultured with RPMI1640 (Takara, Beijing, China) completed culture medium containing 10% FBS and incubated at 37 °C with 5% CO_2 (relative humidity=95%). Culture medium was changed 1−2 days later. Cells were subcultured 3−5 days later and digested with 0.25% trypsinase: 0.03% EDTA (1:1). HEEpiC were cultured with HEEpiC-specific (Takara, Beijing, China) culture medium and the culture procedure was the same as that for TE-1 cells.

Reverse transcription-PCR (RT-PCR)

Total RNA of ESCC tissues, TE-1, TE-10 and HEEpiC were extracted using a RNA extraction kit (Tiangen Biotech, Beijing, China). The purity and integrity of total RNA were tested. cDNAs were reverse transcribed from total RNAs of tissues and cells (2 ng−2 μg) using a reverse transcription kit (Takara, Beijing, China) and then stored at –20 °C. Gene amplifications were performed by taking cDNAs as templates and *GAPDH* as internal reference. Primers for *GAPDH*, *PAR1*, *PAR2* and *PAR4* are shown in Table 1. PCR products were run by 2% agarose gel electrophoresis and observed under ultraviolet light and photographed.

Western blot

Carcinoma tissues and corresponding normal control tissues were randomly selected from three cases. Cultured cells in logarithmic growth were rinsed twice with pre-chilled PBS (4 °C) and then lysed with 6× SDS (0.6 mL) and water-bathed (95 °C) for 15 min. After the polymerization of resolving gel (12%) and staking gel (5%), samples were loaded. Tris-glycine electrophoresis buffer (×l) was poured into the running chamber and loaded samples were run for 1 h at a constant current of 160 V. Then the gel was transferred onto the PVDF membrane for 2 h at a constant current of 250 mA and 4 °C. The blots were removed from the transfer unit and blocked by placing in 3% BSA-TBST blotting solution for 2 h with shaking at room temperature. After washing, the blot was incubated with the primary antibodies for PAR1, PAR2, PAR4 and β-actin over night at 4 °C. After washing, the blot was incubated with secondary antibodies. After another three washes, the blot was revealed via HRP-ECL chemiluminescence detection and scanned using the SynGene scanning system. The illumination densities of the detected protein bands were transferred into optical density values (OD values). The developing strength of each target protein band and its internal reference (β-actin) were determined by OD values. The ratio of the OD values of each target protein band to its internal reference (β-actin) was taken as the result of RT-PCR.

Table 1 Primers and reaction conditions used in RT-PCR

Gene	Primer sequences	NCBI Accession Number	Annealing temperature (°C)	Product (bp)
GAPDH	F: 5′-ATGGGGAAGGTGAAGGTCG-3′ R: 5′-GGGGTCATTGATGGCAACAATA-3′	NM_001101.3	60	308
PAR1	F: 5′-GCCGCCTGCTTCAGTCTGTGC-3′ R: 5′-GGCCAGACAAGTGAAGGAAGC-3′	NM_001992.3	67	648
PAR2	F: 5′-CCATCCAAGGAACCAATAGATC-3′ R: 5′-ATGTCTCCCACCAAGAGCTGCTCA-3′	NM_005242.3	60	643
PAR4	F: 5′-GGCAACCTCTATGGTGCCTA-3′ R: 5′-TTCGACCCAGTACAGCCTTC-3′	NM_003950.2	58	244

Bisulfite genomic sequencing PCR

Genomic DNAs of TE-1, TE-10, HEEpiC and the No. 7 tissue sample were extracted using a genome DNA extraction kit (Takara, Beijing, China) and their concentrations were determined. The CpG sites in *PAR4* were amplified with the MethylCode Bisulfite Conversion Kit (Invitrogen, US). PCR products were eluted and purified with the AxyPrep DNA elution kit (Axygen). Primer sequences were: F-5′-TTTAAGGGTGATTTTAGGAAA GGTTTAGAG-3′and R-5′-ACTATAACCTCAAACTTC CTACCTC-3′. The products of ligation were transformed with DH5α component cells. Transformation products were spread on LB plates (with Ampicillin) and grown overnight. Clones were selected and sent for sequencing.

Statistical analyses

Data were analyzed using SPSS l7.0 (SPSS Inc., Chicago, USA). Correlations between PAR expression and clinical pathologies were tested using Fisher's exact tests. The illumination strength of protein expression is expressed as mean±*SD* and comparisons between expressions were conducted using *t*-tests. Statistical significance was set at $P<0.05$.

RESULTS

mRNA expression of *PAR1*, *PAR2* and *PAR4* and correlation with clinical manifestation

When the expression of *GAPDH* of each sample was at a similar level, the brighter the mRNA band was, the higher the expression level of *PAR* was, and *vice versa*. Among the 28 cases of ESCC, 17 (60%) and 20 (71%) cases were found with increased expression of *PAR1* and *PAR2*, respectively. Totally, 19 (68%) cases were found with decreased *PAR4*. In TE-1 and TE-10, the expression of *PAR1*and *PAR2* was high. The expression of *PAR4* mRNA in TE-1 and TE-10 was lower than in HEEpiC. The expression level of *PAR1*, *PAR2* and *PAR4* mRNA in the tissues and cells from cases 7, 8 and 9 are shown in Figure 1. Combined with clinical pathological data, the expression of *PAR1* in central and low located ESCC was significantly higher than in upper ESCC ($P=0.007$ and $P=0.008$, respectively). The expression of *PAR2* in phase III+IV ESCC was significantly higher than in phase I+II ($P=0.004$). Significantly lower expression of *PAR4* was found in lower ESCC, compared with central and upper ESCC ($P=0.036$). No significant correlation for the expression of *PAR1*, *PAR2* and *PAR4* and patients' gender, sex and distant or lymph node metastases were found (Table 2).

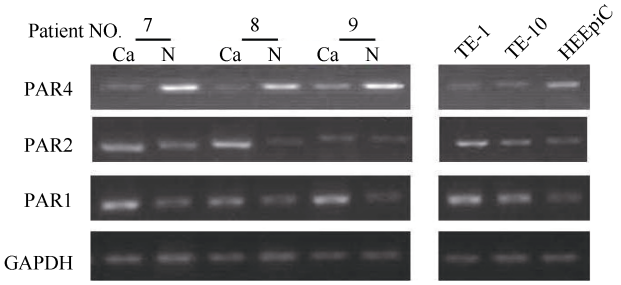

Figure 1 *PAR1*, *PAR2* and *PAR4* mRNA expression in esophageal squamous cell carcinoma and normal control tissues

Protein expression of PAR1, PAR2 and PAR4 in tissues and cells of ESCC

Protein expression was determined by taking β-actin as the internal reference (Figure 2). The expression of PAR1 and PAR2 proteins were increased ($P<0.05$), whereas, those of PAR4 were decreased ($P<0.05$). The light degrees of protein expression of PAR1, PAR2 and PAR4 in sample tissues are shown in Table 3. The expression of PAR1 and PAR2 were high and that of PAR4 was low in TE-1 and TE-10 cells.

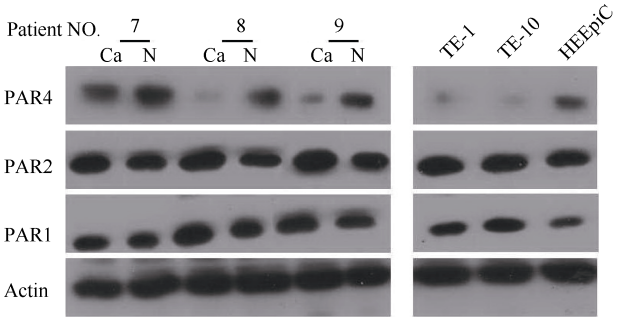

Figure 2 Protein expression of PAR1, PAR2 and PAR4 in tissues and cells of esophageal squamous cell carcinoma

***PAR4* promoter hypermethylation in tissues and cells of ESCC**

The BSP results of the PCR products of CpG sites in *PAR4* are shown in Table 4. In HEEpiC, TE-1 and TE-10, esophageal and normal esophagus tissue from case No. 7, the methylation frequency of the 19 CpG sites in *PAR4* was 35.4%, 95.2%, 83.8%, 62.6% and 48.2%, respectively.

DISCUSSION

PAR2 over-expression is commonly found in malignant tumors. In this study, among 28 cases of ESCC, 20 (71%) cases were found with high *PAR2*

Table 2 Correlation between *PAR1*, *PAR2* and *PAR4* mRNA levels and clinical manifestation of esophageal squamous cell carcinoma

Clinical data	Case numbers (*n*)	*PAR1*			*PAR2*			*PAR4*		
		Decreased	Increased	*P*-value	Decreased	Increased	*P*-value	Decreased	Increased	*P*-value
Age (year)										
≤65	13	5	8	1.000	3	10	0.686	8	5	0.689
>65	15	6	9		5	10		11	4	
Gender										
Male	21	9	12	0.668	4	17	0.142	15	6	0.646
Female	7	2	5		4	3		4	3	
Clinical stage										
Ⅰ+Ⅱ	16	8	8	0.253	7	9	0.088	11	5	1.000
Ⅲ+Ⅳ	12	3	9		1	11		8	4	
Location of the tumor										
Upper	4	4	0	0.016*	0	4	0.549	2	2	0.574
Central and lower	24	7	17		5	16		17	7	
Differentiation										
Well and moderated	17	10	7	0.016*	7	10	0.099	9	8	0.049*
Poor	11	1	10		1	10		10	1	
Distant metastasis										
Positive	1	0	1	1.000	0	1	1.000	1	0	1.000
Negative	27	11	16		8	19		18	9	
Lymph node metastasis										
Positive	11	4	7	1.000	2	9	0.419	8	3	0.704
Negative	17	7	10		6	11		11	6	

*: $P<0.05$.

Table 3 Gray values of PAR expression in sample tissues

Gene	Cancer tissue	Normal tissue	*t*	*P*
PAR4	0.18±0.05	0.42±0.12	9.77	0.0001**
PAR2	0.84±0.28	0.69±0.25	7.97	0.039*
PAR1	0.74±0.31	0.58±0.21	2.26	0.027*

*: $P<0.05$; **: $P<0.01$.

mRNA expression. The expression of *PAR2* mRNA was also increased in TE-1 and TE-10 cells. The gray value indicates that PAR2 protein expression is increased in ESCC (P=0.039). PAR2 can be activated by trypsinase, human mast cell tryptase (MCT), coagulation factor VIIa and tissue factor complex (Caruso et al, 2006; Uusitalo-Jarvinen et al, 2007). The *in vitro* synthesized SLIGKV by the degradation of PAR2 can also activate PAR2 (Déry et al, 1998). Activated PAR2 may release vascular endothelial growth factor (VEGF), Interleukin-6 (IL-6) and IL-8 of tumor cells and thereafter promote the generation and invasion of novel vessels by malignant tumors (Knecht et al, 2007; Matej et al, 2007). Activated PAR2 may also increase the reverse activity of epidermal growth_factor (EGF) and the release of transformation growth factor α (TGFα), and thus promote the proliferation of gastrointestinal cancer cells in stomach cancer, colon cancer, pancreatic cancer and ESCC (Darmoul et al, 2004; Fujimoto et al, 2006; Yada et al, 2005).

PAR1 and PAR4 are both thrombin receptors. Among the 28 cases of ESCC examined in the present study, 17 (60%) cases were found with high expression of *PAR1* mRNA; however, 19 (68%) cases were found with low expression of *PAR4* mRNA. The expression of *PAR1* mRNA was increased in TE-1 and TE-10 cells, whereas, that of *PAR4* was decreased. The gray value indicates that PAR1 protein expression was increased in ESCC (P=0.027) and that of PAR4 was significantly decreased (P=0.0001). High PAR1 expression was found in ESCC, particularly in central and lower (P=0.016) and poorly differentiated ESCC (P=0.049), whereas, the low expression of PAR4 was found in ESCC, particularly in

poorly differentiated ESCC.

Table 4 Methylation frequency of the 19 CpG sites in *PAR4* of cells and tissue of esophageal squamous cell carcinoma

CpG position	Gene	3	12	41	68	95	124	195	215	227	259	275	292	327	330	332	341	345	350	377	Total
Me-CpG (%)	HEEPIC	18	73	0	64	27	55	73	9	46	0	46	55	27	0	0	0	73	55	55	35.4
	TE-1	100	100	90.9	100	100	81.8	100	30	100	100	82	91	100	100	91	91	100	100	100	95.2
	TE-10	82	100	100	100	100	100	100	91	100	100	46	73	55	55	100	100	100	55	30	83.8
	Cancer tissue	64	46	30	46	64	9	64	100	91	100	100	100	91	91	9	46	46	30	64	62.6
	Normal tissue	91	64	9	55	64	30	55	9	55	100	100	9	0	55	55	30	64	30	46	48.2

To investigate the underlying mechanisms of PAR4 and ESCC, methylation of the *PAR4* promoter in tissues and cells of ESCC was evaluated. The results show that the methylation frequencies of *PAR4* promoters in sample tissues of ESCC (62.6%) and TE-1 and TE-10 (83.8% and 95.2%, respectively) were both high. Together with the fact that the expression of *PAR4* mRNA and protein is lower in tissues and cells of ESCC, these findings indicate that the methylation frequency of *PAR4* promoters may play a role in its expression in ESCC.

Kawabata et al (1999) found the relaxation induced by PAR4 aggravates duodenal-gastric-esophageal reflux, and trypsinase within the reflux liquid may activate PAR4, thus promoting the incidence of ESCC. Han et al (2011) found that the promoting effects of PAR4 on anti-angiogenesis factors, including endostatin, thrombostondin 1, α2 macroglobulin, plasminogen activator, angiostatin and enzyme inhibitors may remarkably inhibit the generation of novel vessels and tumors. Human blood platelets only express PAR1 and PAR4. Activated PAR4 inhabits the release of VEGF but promotes the expression of endostatin, whereas, the effects of activated PAR1 are the opposite (Ma et al, 2005). Longitudinal gastrointestinal smooth muscles are intensified by PAR1 but relaxed by PAR4. PAR4 also prevents over intension in smooth muscles induced by PAR1 and thrombin (Lan et al, 2000). Cunningham et al (2012) found that similar interactions between PAR2 and PAR4 may play a role in locating receptors and cellular signal transduction.

In sum, here we investigated the expression characteristics of PAR1, PAR2 and PAR4 in ESCC tissues to broaden our understanding of PARs in ESCC. As G-protein-coupled receptors, the complicated biological distribution and function of PAR1, PAR2 and PAR4 remain unclear and their activity and interactions require further attention.

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