PAR1 and PAR4 exert opposite effects on tumor growth and metastasis of esophageal squamous cell carcinoma via STAT3 and NF-κB signaling pathways

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Primary research

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Abstract

Background

Esophageal carcinogenesis is a multifactorial process in which genetic and environmental factors interact to activate intracellular signals, leading to the uncontrolled survival and growth of esophageal squamous cell carcinoma (ESCC) cells. The intracellular pathways of ESCC cells could be regulated by proteinase activated-receptors (PARs), which are comprised of four receptors (i.e., PAR-1, PAR-2, PAR-3, and PAR-4). Therefore, the function and possible mechanism of PAR1 and PAR4 in the progression of ECSS were explored.

Methods

First, we detected the expression levels of PAR1 and PAR4 in 27 cases of ESCC tissue specimens and cell lines by RT-qPCR, IHC and western blot. Meanwhile, the correlation between PAR1/PAR4 expression level, clinicopathological characteristics, and disease free survival were analyzed. Then, we constructed PAR1/PAR4 knockdown cell models and investigated the role of PAR1/PAR4 knockdown on the proliferation, apoptosis, changes of calcium flow, and metastasis of ESCC cell via MTT, flow cytometry, transwell and wound healing assays in vitro. Further, an experimental metastasis model in vivo was established to explore the role of stable PAR1/PAR4 knockdown on the growth and metastasis of ESCC cells. Finally, the role of nSMase2 in the activation of NF-κB induced by PAR4 and the role of NF-κB and STAT3 signaling pathways in the PAR1/PAR4-mediated tumor promoting or suppressive functions were measured by immunoprecipitation, western blot and immunofluorescence.

Results

The integrated results demonstrated the expression levels of PAR1 and PAR4 are inversely proportional in ESCC. PAR1 could potently enhance tumor growth and metastasis, while PAR4 had an inhibitory effect. Further, the co-activation of STAT3 and NF-κB is involved in the PAR1 activation-induced tumor promoting effect, while only NF-κB participates in the PAR4 activation-induced tumor inhibitory effect in ESCC. To be specific, FAK/PI3K/AKT/STAT3/NF-κB mediated PAR1 activation-induced tumor promoting effect and nSMase2/MAPK/NF-κB mediated PAR4 activation-induced tumor inhibitory effect.

Conclusions

Overall, the study provided new insights into the potential implication of PAR1 and PAR4 in the pathogenesis of ESCC. Besides, FAK/PI3K/AKT/STAT3/NF-κB and nSMase2/MAPK/NF-κB pathways may be novel targets for regulating tumor growth and cancer metastasis in ESCC patients.
Esophageal cancer (EC) is one of the most aggressive gastrointestinal cancer.[1] East Asian countries, including China, are the areas of high incidence of esophageal squamous cell carcinoma (ESCC).[2] Due to the lack of early clinical symptoms, ESCC is often diagnosed during its advanced stages and becomes a highly aggressive malignancy with a poor prognosis.[3] Therefore, it is crucial to find a potentially valuable and early diagnostic indicator.

Many studies have demonstrated that the hemostatic components may participate in the progress of cancer development.[4–6] Coagulant factors, like tissue factor (TF) and thrombin, may be generated in the tumor microenvironment and can induce cell signaling by activating protease-activated receptors (PARs).[7, 8] Protease-activated receptors (PARs) is a unique family of G-protein coupled receptors, which plays an important role in vascular physiology, neural tube closure, hemostasis, and inflammation.[9] Some of their agents are considered to promote cancer metastasis.[10, 11] There are four members of PARs in humans, PAR1, PAR2, PAR3 and PAR4, sharing the common mechanism of activated by proteolysis.[12] Currently, studies have shown that PAR1 is positively expressed in 68.2% of tumor tissues derived from EC patients but not in normal esophageal squamous epithelium. PAR1 overexpression is significantly related to tumor metastasis (TNM) stage and regional lymph node involvement.[13] High expression of PAR2 in ESCC are also reported.[14] On the contrary, the expression of PAR4 was reduced in esophageal squamous cancer. PAR1 and PAR4 are thrombin receptors and share the same ligand and activation mechanism, but their expression levels are inversely proportional in ESCC cells. This discovery aroused our interest in exploring the role of PAR1 and PAR4 in the development of ECSS, which has not been clearly explored yet.

In this study, we found that PAR1 induced the development of ESCC through the co-activation pathway of STAT3 and NF-κB, and the inhibition of the AKT pathway also played an important role. On the contrary, PAR4 had an inhibitory effect on the development of ESCC by activating ERK and NF-κB rather than STAT3. However, this study provides new insights into the potential implication of PAR1 and PAR4 in the pathogenesis of ESCC.

Methods

Cells and reagents

Human esophageal epithelial cells (HEEC) cells were purchased from ScienCell and were maintained according to standard protocol. EC109, Kyse150, Kyse140 and TE-1 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI-1640 (Cat.no.72400120, Gibco, CA, USA) containing 10% FBS (fetal bovine serum) and 1% penicillin-streptomycin. Tumor cells are cultured in a 5% (v/v) CO₂ humidified incubator at 37°C.

Clinical Samples

A total of 27 tumor tissue specimens without treatment with chemotherapy were collected from Hospital of Zhengzhou University. The whole investigation was...
approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University and written consents were provided by all patients. The clinical diagnosis was confirmed by two experienced pathologists without discrepancy.

**Animals**

C57BL/6J mice were from JSJ laboratories (China) and were bred and housed at The First Affiliated Hospital of Zhengzhou University animal care facility. Animal experiments were conducted in mice using protocols approved by the IACUC of The First Affiliated Hospital of Zhengzhou University Six- to eight-week-old mice were applied in the study, and were acclimated and housed in a clean grade room at 21 ± 1°C and 60 ± 5% humidity, under a 12 hours light/12 hrs dark cycle with free access to water and food in a specific pathogen-free environment. Animal husbandry protocols were followed where animals were monitored daily according to humane endpoint guidelines by experimental staff and independently by animal husbandry staff, including in-house veterinarians. All animals were assessed to be healthy and free of disease prior to experiments. Mice were euthanized with an intraperitoneal injection of 150 mg/kg sodium pentobarbital 50 days after subcutaneous injection with tumor cells, and then the heartbeat of the mice was examined.

**The construction of knockdown and overexpression plasmids**

PAR1 or PAR4 short hairpin RNA (shRNA, synthesized by Gene Pharma, CN) were utilized to transfect into tumor cells for gene knockdown. Sequences of shRNA targeting PAR1, PAR4, Akt were designed and cloned into the pSicoR-GFP vector (Addgene, Cambridge, MA).

For overexpression, the gRNA for PAR1 or PAR4 was designed at the following website ([http://sam.genome-engineering.org/database/](http://sam.genome-engineering.org/database/)). The PAR1/PAR4 overexpressing gRNA was designed to target the sequence. Then the gRNA oligo was inserted into lenti gRNA (MS2)-puro plasmid, by the process of Oligo annealing, digestion with BsmBI, ligation, transformation and plasmid extraction, the PAR1/PAR4 plasmids were obtained and were transfected into cells. 72 hrs after transfection, the lentivirus for PAR1/PAR4-overexpression was collected and filtered through 0.45 µm filters. After infection with the virus for 24 hrs, the medium with virus was removed and the targeted cancer cells were retreated with fresh culture media for further analysis.

**Spontaneous metastasis model**

To investigate the role of PAR1/PAR4 in ESCC metastasis, 6-8 weeks old C57BL/6J mice were randomly divided into 6 groups. Each group had 6 mice. The mice were subcutaneously injected with 1× 10^5 sham-transfected TE-1 cells (NC), PAR1 or PAR4 knock down TE-1 (PAR1 KD/PAR4 KD) and PAR1 or PAR4 overexpressed TE-1 (PAR1 OE/PAR4 OE) respectively. After 50 days, the mice were killed, then the metastatic nodules on the lung surface were counted and the primary tumor volume was recorded.
To investigate the role of STAT3 on PAR1 activating tumor metastasis, 6-8 weeks old BALB/c mice were randomly divided into 3 groups. Each group had 6 mice. The mice were subcutaneously injected with 1×10^5 sham-transfected Kyse140 cells. PAR1 AP and STAT3 inhibitor S3I-201 were mixed and injected via tail vein. After 50 days, the mice were killed, then the metastatic nodules on the lung surface were counted and the primary tumor volume was recorded.

**Immunofluorescence assay**

Tumor cells were blocked with 3% PBS and 5% BSA (Sigma) for 1 hour at room temperature (RT), incubated in blocking solution overnight with anti-p-STAT3 antibody (Abcam, ab5694) anti-STAT3 antibody (Abcam, ab5694) anti-p-NF-κB antibody (Abcam, ab5694) for 1 hour at RT. After washing with PBS, the cells were incubated with Cy3-anti rabbit IgG or FITC-anti rabbit IgG for 60 min. The fluorescence images were captured using confocal microscope (Nikon).

**Western blot**

Cells were lysed with cold RIPA buffer plus phosphatase and protein inhibitors and centrifuged at 12,000 rpm for 30 min to remove cell debris. The total protein concentrations were determined by the Bradford method. 40 μg protein were loaded, separated on SDS-PAGE and electro-transferred to a polyvinylidene difluoride membrane. Membranes were blocked with 5% bovine serum albumin (BSA) in a Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.6) for 60 min and then blotted overnight at 4°C with respective primary antibodies. After incubating with HRP-conjugated second antibody for 60 min at RT, the antibody binding was detected using Tanon Imaging System (5200S).

**Flow cytometry**

Apoptosis assay was quantitated by a Becton-Dickinson FACS Canto II instrument equipped with Annexin V-FITC Apoptosis Detection Kit (C1062M, Beyotime, China). The subsequent steps were carried out by following the manufactures’ instructions.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT Assay)**

Tumor cells were treated in indicated way. The culture medium of cells were added with 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) and incubated at 37°C for 4 hrs; 100 μl dimethyl sulfoxide was then added. The optical density (OD) was measured at both 492 nm and 630 nm wavelength; Cell viability was calculated according to the following formula: cell viability = [OD value of treatment group-OD value of background] / [OD value of control group-OD value of background] × 100%.

**Real-time RT-PCR**

According to the manufacturers’ instructions, total RNA was extracted from cell lines with the TRIzol reagent (Invitrogen, USA), cDNA was synthesized with the PrimeScript RT Reagent Kit (TransGen Biotech,
China). qRT-PCR analysis was performed with the SYBR Green Premix Ex Taq (Takara, China). The total RNA levels were normalized with GAPDH. Forward (F) and reverse (R) primers were as followed: GAPDH-F, 5'-GAC TGACTGCCTCTA-3'; GAPDH-R, 5'-GGAGTGCGTGCTGCTG-3'; PAR1-F: 5' -ATGGACA T T TATAG T ∀ GGA -3'; PAR1-R: 5' ∀ GGTATCTGA T TGATATV -3'; PAR4-F: 5' -CGATCTGCTGTC GC TCGG -3'; PAR4-R: 5' -TAGCACCCV CTCTGCTC -3'. All of the primers were synthesized by Sunny Bio Co..

**Transwell assay and wound healing**

For Transwell assay, cultured ESCC cells were added to upper chambers (10^4 cells per well) of 8µm-24 well Transwell culture dishes with 10% FBS culture medium in lower chambers. After 24 hours, cells were rinsed with calcium free PBS and fixed with methanol, then stained with crystal violet. Tumor cells passed through the membranes were evaluated in five randomly selected fields, then dissolved in methanol and quantified by microplate reader OD 405 nm. The wound healing was carried out as previously described. [15]

**Bioinformatics analysis**

The data used for Disease Free Survival (DFS) were provided from The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/docs/publications/tcga/). The Kaplan–Meier method with the log-rank test was used to calculate the DFS rate for comparison between different groups. A corrected P-value < 0.05 was adopted as the standard for judging statistically significant enrichment of cluster genes.

**Statistical analysis**

Statistical analysis was performed using Prism 6 software. All experiments were carried out at least three times, and the results were presented as the mean ± standard deviation. Statistical significance between two groups was assessed by using the one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. P-values < 0.05 were considered statistically significant.

**Results**

**The expression levels of PAR1 and PAR4 are inversely proportional in ESCC**

We clinically collected 19 cases of ESCC tissues and paired normal adjacent tissues. The results of qPCR and IHC showed that the expression of PAR1 in tumors was higher, while the expression of PAR4 was lower, than that in normal adjacent tissues (Figure 1A, B). The similar results were also found with western blot and RT-PCR (Figure 1C, D). Next, we analyzed the expression of PAR1 and PAR4 in ESCC tissues and normal adjacent tissues according to the data collected from TCGA database. The bioinformatic results from GEPIA (http://gepia.cancer-pku.cn/) indicated that the levels of PAR4 are lower, while the levels of PAR1 are significantly higher in tumor tissues than in normal tissues (Figure 1E). Then,
the expression of PAR1 and PAR4 was detected in Human normal esophageal epithelial cells (HEEC) and ESCC cell lines EC109, Kyse140, Kyse150 and TE-1. Western blot results suggested that PAR4 was significantly downregulated in all the tumor cell lines, while PAR1 was overexpressed in three of the tumor cell lines, compared with that of HEEC cells. (Figure 1F).

**PAR1 promoted the growth and metastasis of ESCC cell line, while PAR4 had an inhibitory effect in vitro**

To explore the impact of PAR1/PAR4 on cancer metastasis in ESCC, we investigated clinical ESCC tissue specimens with qPCR and IHC assays. As shown in Figure 2A and 2B, compared with non-metastatic patients, the expression of PAR1 in patients with metastasis had increased by 50%, while the expression of PAR4 decreased. In addition, tumor tissues derived from patients in late stage III-IV (TNM) showed lower PAR4 levels and higher PAR1 levels, compared with patients in stage II (Supplementary Figure 1A, B). The similar results were also demonstrated with western Blot (Figure 2C), indicating that PAR1 and PAR4 may play the opposite roles in the development of ESCC, and PAR1 may have great impact on cancer metastasis in ESCC. Then, we investigated the correlation between disease free survival (DFS) and the levels of PAR1 and PAR4. The Kaplan-Meier survival analysis revealed that higher PAR1 expression was associated with poor survival, while higher PAR4 expression was linked with prolonged survival of ESCC patients (Figure 2D).

To further explore the role of PAR1 and PAR4 in ESCC, we transiently knockdown PAR1 and PAR4 in HEEC, Kyse140 and TE-1. Western blot results demonstrated effective silencing of these two genes (Figure 2E). The effect of PAR1 and PAR4 knockdown on cell proliferation was detected by MTT assay. As shown in Figure 2F and 2G, PAR1 knockdown significantly inhibited the proliferation of Kyse-140, PAR4 knockdown had the opposite effect (Figure 2F, G) and the results of TE-1 and HEEC cell lines were shown in Supplementary Figure 2A. Flow cytometry and western blot were applied to detect the expression levels of apoptosis-related molecules. The results showed that PAR1 knockdown significantly inhibited the apoptosis of ESCC cell line, which is opposite of PAR1 knockdown (Supplementary Figure 2C, D).

Meanwhile, by comparing the protein levels of PAR1 and PAR4 in patients’ tumor tissues whose diameters were larger than or less than 1000 square millimeters, we found that the expression of PAR1 was upregulated in larger tumors, while that of PAR4 was downregulated (Supplementary Figure2B). Then, the effect of PAR1 and PAR4 on cancer metastasis was detected by wound healing and transwell assays. Results revealed that the migration of PAR1 knockdown cell line was suppressed while the migration of PAR4 knockdown cell line was promoted, and the results of Kyse140 cell line were shown in Figure 2H and 2I, and the results of TE-1 were shown in Supplementary Figure 2E and 2F).

Next, we detected the changes of calcium flow, and found that PAR1 and PAR4 AP could activate Kyse140 cell line (Figure 3A). PAR1 activation significantly enhanced cell growth in Kyse140 and TE-1 cells. Meanwhile, PAR4 activation had a negative effect on cell growth (Figure 3B). Then, we found that PAR1 AP could slightly inhibit tumor cell apoptosis and PAR4 AP could significantly promote tumor cell apoptosis (Figure 3C and 3D). Besides, PAR1 activation also significantly enhanced the migration ability of tumor cells in vitro, while PAR4 activation had the inhibitory effect (Figure 3E, F, G). Taken together,
these data suggested that PAR1 could potently enhance tumor cell growth and metastasis, while PAR4 had an inhibitory effect in vitro.

**PAR1 promoted the proliferation and metastasis of ESCC cell line, while PAR4 had an inhibitory effect in vivo**

In order to further demonstrate the role of PAR1 and PAR4 in tumor metastasis *in vivo*, we established an experimental metastasis model with female C57BL/6J mice. First, the expression of PAR1 and PAR4 in TE-1 cell line was knocked down by lentivirus infection, and was overexpressed by CRISPR-SAM. The efficiency of knockdown and overexpression was confirmed by western blot (Figure 4A, D). Consistent with the *in vitro* observations, as shown in Figure 4B-F, the PAR1 knockdown cells markedly suppressed the growth of tumor compared with the control mice. The similar results were also found in the mice injected with PAR4 overexpression cells. This finding was also confirmed by the number of pulmonary nodules on the lung surface. PAR1 knockdown significantly reduced the number of metastatic nodules while the PAR1 overexpression markedly increased the number of nodules (Figure 4G). Besides, PAR4 overexpression significantly inhibited the pulmonary metastasis (Figure 5I). The similar results were also found with H&E staining, by which we observed the metastatic lesions under a microscope (Figure 5H, J).

**The co-activation of STAT3 and NF-κB is involved in the PAR1 activation-induced tumor promoting effect, while only NF-κB participates in the PAR4 activation-induced tumor inhibitory effect**

Several studies illustrated the specificity of NF-κB/STAT3 pathway activation—STAT3 and NF-κB jointly regulate several oncogenes and inflammatory genes.[16] When NF-κB is activated alone, it exerts an tumor suppressor. Recent studies have focused on the relationship between PAR1/PAR4 and NF-κB/STAT3 signaling pathway, which enlightened us that the reason why PAR1 and PAR4 had completely opposite effects on ESCC progress might be attributed to the distinct activation of STAT3 and NF-κB in ESCC cell lines. First, western Blot was applied to investigate the impact of PAR1 AP on STAT3 and NF-κB levels in the nucleus. As shown in Figure 5A, PAR1 activation by AP obviously increased the protein levels of p-STAT3 and p-NF-κB in the nucleus, which was reversed by PAR1 knockdown. We also obtained the above discovery under a confocal microscopy (Figure 5B). Then, We detected the impact of PAR4 AP on STAT3 and NF-κB levels in the nucleus. As shown in Figure 5C, the activation or knockdown of PAR4 had no effect on the expression level of p-STAT3 in the nucleus, but the activation of PAR4 significantly enhanced the expression level of p-NF-κB in the nucleus, which was reversed by PAR4 knockdown. Similarly, we also found that in PAR4 AP-activated ESCC cells, STAT3 was mostly distributed in the cytoplasm, while p-NF-κB was in the nucleus (Figure 5D). Then, we applied STAT3 inhibitor S3I-201 to explore whether STAT3 activation and the nuclear translocation of p-STAT3 have effects on the PAR1 activating-induced cancer promoting effect. The western blot and immunofluorescence results showed that S3I-201 could inhibit the expression of p-STAT3 in the nucleus induced by PAR1 AP (Figure 5E, F). Meanwhile, S3I-201 significantly reversed the effect of PAR1 AP on the enhanced proliferation of ESCC cells, but had no effect on the suppressive proliferation of ESCC cells induced by PAR4 AP (Figure 5G). In addition, the results of *in vivo* metastasis model showed that compared with the group injected with
PAR1 AP alone, the number of pulmonary metastatic nodules in PAR1AP and S3I-201 co-injection group was significantly reduced (Figure 5H).

**nSMase2/MAPK/NF-κB mediated PAR4 activation-induced tumor inhibitory effect**

Based on the preliminary experiments and literature, we put forward a hypothesis that nSMase2 might play a role in the activation of NF-κB induced by PAR4 AP. Kyse140 cells were immunoprecipitated with anti-PAR1 or anti-PAR4 antibodies, followed by immunoblotting with anti-nSMase2 antibody. As shown in Figure 6A, nSMase was directly associated with PAR4 but not PAR1. We also speculated whether the different effects of PAR1 and PAR4 on ESCC were related to the activation of different signaling pathways. Then, western blot detected the expression levels of p-p38 MAPK under the conditions of PAR1/PAR4 activation or knockdown. The results showed that the expression of p-p38 MAPK was upregulated by both PAR1 and PAR4 activation (Figure 6B). Then, flow cytometry was applied to demonstrate that the levels of p-IKKβ were increased by PAR1 or PAR4 activation. However, we found that 3-OMS (a nSMase inhibitor) almost completely abolished IKKβ phosphorylation induced by PAR4 AP but did not affect phosphorylation induced by PAR1-AP (Figure 6C).

Next, we explored the FAK phosphorylation after PAR1 AP or PAR4 AP treatment. As shown in Figure 6D, PAR1 activation, but not PAR4 activation, downregulated the levels of p-FAK. Meanwhile, PAR1 activation had a significant inhibitory effect on the expression of PI3K and p-Akt, while PAR4 exerted little effect (Figure 6E). MTT assays demonstrated that nSMase2 activation was involved in PAR4-mediated tumor inhibitory effect and nSMase2 inhibitor 3-OMS weakened this effect induced by PAR4 AP (Figure 6F). To sum up, PAR4 activation induced nSMase2/MAPK pathway, and IKKβ phosphorylation promoted NF-κB phosphorylation and nuclear translocation, and then produced the anti-cancer effect.

**FAK/PI3K/AKT/STAT3/NF-κB mediated PAR1 activation-induced tumor promoting effect**

In order to further explore whether AKT played a role in PAR1-induced tumor promoting effect, we first constructed AKT knockdown Kyse140 cell line (Figure 6G). Immunofluorescence results have shown that PAR1 AP induced the distribution of p-STAT3 in the nucleus, while the distribution of p-NF-κB in the nucleus was slightly affected (Figure 6H). To sum up, PAR1 activation inhibited FAK phosphorylation and further suppressed PI3K/AKT, thereby inducing the nucleus translocation of p-STAT3, and then exerted an tumor promoting effect.

**Discussion**

PARs have been demonstrated to regulate tumor growth, invasion and metastasis in a variety of malignant tumors.17–19 PAR1, which is widely expressed in human cancers, promotes the transformation and adhesion of pancreatic cancer cells and the invasion and metastasis of oral adenocarcinoma, colon cancer and breast cancer cells.20–26 PAR2 is closely related to the growth and invasion of nasopharyngeal cancer, breast cancer, gastric cancer, colon cancer, prostate cancer and
prognostic markers because their expression levels are closely linked with the stage of tumor. However, the role of PAR1 in the development of ESCC is still unclear. The latest study has shown that compared with matched non-cancerous tissues, the expression of PAR4 in gastric cancer tissues is significantly reduced, especially in tumors with lymph node or poorly differentiated tumors. Jiang et al. have reported that the down-regulated expression of PAR4 was found in lung adenocarcinoma, and the decrease of PAR4 levels was associated with a more aggressive clinical phenotype, suggesting that PAR4 may exert a tumor suppressor in lung adenocarcinoma. However, PAR4 is overexpressed in colorectal cancer and liver cancer, and the overexpression of PAR4 contributes to the proliferation of cancer cells. In addition, PAR4 agonists can induce calcium influx and promote the proliferation of colon cancer cells through ErbB2 transcription activation and Src kinase pathway.

In our study, we first collected and detected clinical samples and found that PAR4 levels were decreased and PAR1 levels were increased in esophageal squamous cancer. Besides, the levels of PAR4 and PAR1 were related to tumor size and distant metastasis. Then, we noted that PAR4 exerts an tumor suppressor and PAR1 has the opposite effect in ESCC which is confirmed by both in vitro and in vivo experiments. PAR1 and PAR4 might be a potential biomarker for ESCC diagnosis and prognosis.

Moreover, we explored the role and mechanism of STAT3/NF-κB pathway in the PAR1/PAR4 activation-induced tumor promoting/inhibitory effect. The experimental results revealed that PAR1 activation induced the nuclear translocation of STAT3 and NF-κB while PAR4 activation could only induce the nuclear translocation of NF-κB. Then, we further confirmed the role of the FAK/PI3K/AKT pathway in PAR1-activation induced nucleus translocation of STAT3 and the role of nSMase2/MAPK pathway in PAR4-activation induced nucleus translocation of NF-κB. In addition, it is well known that PAR1 and PAR4 are calcium channel proteins. We found that PAR1 and PAR4 activation induced significant changes in calcium flow of ESCC cells. Therefore, the intracellular pathways that mediate PAR1 and PAR4 activation-induced changes in calcium flow are worth further investigating in the future.

Conclusions

In brief, as shown in Fig. 7, nSMase2/MAPK/NF-κB mediated PAR4 activation-induced tumor inhibitory effect while FAK/PI3K/AKT/STAT3/NF-κB mediated PAR1 activation-induced tumor promoting effect in ESCC.

Declarations

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Authors’ contributions
SZ designed the project, applied for the funds and approved the submission of the manuscript. JZ, GYJ, XDL, ZFH, DLL, CYZ and DYZ conducted in vitro experiments. JZ, GYJ and XDL analyzed the data; KW and YY conducted animal experiments. JZ wrote the manuscript.

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**Availability of data and material**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Declarations**

**Ethics Approval and consent to participate**

Twenty-seven clinical ESCC samples were collected with ethical approval from the research ethics committees of The First Affiliated Hospital of Zhengzhou University.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declared no conflicts of interest with respect to the research, authorship and publication of this article.

**References**

1. Watanabe M, Otake R, Kozuki R, Toihata T, Takahashi K, Okamura A, Imamura Y: Recent progress in multidisciplinary treatment for patients with esophageal cancer. *Surgery today* 2020, 50(1):12-20.

2. Domper Arnal MJ, Ferrández Arenas Á, Lanas Arbeloa Á: Esophageal cancer: Risk factors, screening and endoscopic treatment in Western and Eastern countries. *World journal of gastroenterology* 2015, 21(26):7933-7943.

3. Huang FL, Yu SJ: Esophageal cancer: Risk factors, genetic association, and treatment. *Asian journal of surgery* 2018, 41(3):210-215.

4. Zacharski LR, Henderson WG, Rickles FR, Forman WB, Cornell CJ, Jr., Forcier RJ, Edwards RL, Headley E, Kim SH, O'Donnell JE et al. Effect of warfarin anticoagulation on survival in carcinoma of the lung, colon, head and neck, and prostate. Final report of VA Cooperative Study #75. *Cancer* 1984, 53(10):2046-
5. Nierodzik ML, Karpatkin S: **Thrombin induces tumor growth, metastasis, and angiogenesis: Evidence for a thrombin-regulated dormant tumor phenotype.** *Cancer cell* 2006, 10(5):355-362.

6. Zigler M, Kamiya T, Brantley EC, Villares GJ, Bar-Eli M: **PAR-1 and thrombin: the ties that bind the microenvironment to melanoma metastasis.** *Cancer research* 2011, 71(21):6561-6566.

7. Austin KM, Covic L, Kuliopulos A: **Matrix metalloproteases and PAR1 activation.** *Blood* 2013, 121(3):431-439.

8. Yang E, Boire A, Agarwal A, Nguyen N, O’Callaghan K, Tu P, Kuliopulos A, Covic L: **Blockade of PAR1 signaling with cell-penetrating pepducins inhibits Akt survival pathways in breast cancer cells and suppresses tumor survival and metastasis.** *Cancer research* 2009, 69(15):6223-6231.

9. Sedda S, Marafini I, Caruso R, Pallone F, Monte Leone G: **Proteinase activated-receptors-associated signaling in the control of gastric cancer.** *World journal of gastroenterology* 2014, 20(34):11977-11984.

10. Fujimoto D, Hirono Y, Goi T, Katayama K, Yamaguchi A: **Prognostic value of protease-activated receptor-1 (PAR-1) and matrix metalloproteinase-1 (MMP-1) in gastric cancer.** *Anticancer research* 2008, 28(2a):847-854.

11. Suen JY, Barry GD, Lohman RJ, Halili MA, Cotterell AJ, Le GT, Fairlie DP: **Modulating human proteinase activated receptor 2 with a novel antagonist (GB88) and agonist (GB110).** *British journal of pharmacology* 2012, 165(5):1413-1423.

12. Ossovskaya VS, Bunnett NW: **Protease-activated receptors: contribution to physiology and disease.** *Physiological reviews* 2004, 84(2):579-621.

13. Peng HH, Zhang X, Cao PG: **MMP-1/PAR-1 signal transduction axis and its prognostic impact in esophageal squamous cell carcinoma.** *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas* 2012, 45(1):86-92.

14. Wang X, Liu HT, Li S, Li K, Lin N, Fan QX, Zheng YL: **Prognostic value of protease-activated receptor 2 expression in oesophageal squamous cell carcinoma.** *The Journal of international medical research* 2010, 38(4):1381-1388.

15. Cai AQ, Landman KA, Hughes BD: **Multi-scale modeling of a wound-healing cell migration assay.** *Journal of theoretical biology* 2007, 245(3):576-594.

16. Cong Y, Cui Y, Zhu S, Cao J, Zou H, Martin TA, Qiao G, Jiang W, Yu Z: **Tim-3 promotes cell aggressiveness and paclitaxel resistance through NF-κB/STAT3 signalling pathway in breast cancer cells.** *Chinese journal of cancer research = Chung-kuo yen cheng yen chiu* 2020, 32(5):564-579.

17. Jahan I, Fujimoto J, Alam SM, Sato E, Sakaguchi H, Tamaya T: **Expression of protease activated receptor 2 in relation to cell proliferation and advancement of uterine endometrial cancers.** *Oncology*
18. Johnstone RW, See RH, Sells SF, Wang J, Muthukkumar S, Englert C, Haber DA, Licht JD, Sugrue SP, Roberts T et al: A novel repressor, par-4, modulates transcription and growth suppression functions of the Wilms' tumor suppressor WT1. *Molecular and cellular biology* 1996, 16(12):6945-6956.

19. Fujimoto D, Hirono Y, Goi T, Katayama K, Matsukawa S, Yamaguchi A: The activation of proteinase-activated receptor-1 (PAR1) promotes gastric cancer cell alteration of cellular morphology related to cell motility and invasion. *International journal of oncology* 2013, 42(2):565-573.

20. Tekin C, Aberson HL, Waasdorp C, Hooijer GKJ, de Boer OJ, Dijk F, Bijlsma MF, Spek CA: Macrophage-secreted MMP9 induces mesenchymal transition in pancreatic cancer cells via PAR1 activation. *Cellular oncology (Dordrecht)* 2020, 43(6):1161-1174.

21. Queiroz KC, Shi K, Duitman J, Aberson HL, Wilmink JW, van Noesel CJ, Richel DJ, Spek CA: Protease-activated receptor-1 drives pancreatic cancer progression and chemoresistance. *International journal of cancer* 2014, 135(10):2294-2304.

22. Segal L, Katz LS, Shapira H, Sandbank J, Geras-Raaka E, Gershengorn MC, Oron Y: PAR-3 knockdown enhances adhesion rate of PANC-1 cells via increased expression of integrinav and E-cadherin. *PloS one* 2014, 9(4):e93879.

23. Jia Y, Zhang S, Miao L, Wang J, Jin Z, Gu B, Duan Z, Zhao Z, Ma S, Zhang Wet al: Activation of platelet protease-activated receptor-1 induces epithelial-mesenchymal transition and chemotaxis of colon cancer cell line SW620. *Oncology reports* 2015, 33(6):2681-2688.

24. Wang Y, Liao R, Chen X, Ying X, Chen G, Li M, Dong C: Twist-mediated PAR1 induction is required for breast cancer progression and metastasis by inhibiting Hippo pathway. *Cell death & disease* 2020, 11(7):520.

25. McAuley JR, Bailey KM, Ekambaram P, Klei LR, Kang H, Hu D, Freeman TJ, Concel VJ, Hubel NE, Lee J, et al: MALT1 is a critical mediator of PAR1-driven NF-κB activation and metastasis in multiple tumor types. *Oncogene* 2019, 38(49):7384-7398.

26. Boire A, Covic L, Agarwal A, Jacques S, Sherifi S, Kuliopulos A: PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells. *Cell* 2005, 120(3):303-313.

27. Li Z, Bian L, Li Y, Liang YJ, Liang HZ: Expression of protease-activated receptor-2 (PAR-2) in patients with nasopharyngeal carcinoma: correlation with clinicopathological features and prognosis. *Pathology, research and practice* 2009, 205(8):542-550.

28. Matej R, Mandáková P, Netíková I, Poucková P, Olejár T: Proteinase-activated receptor-2 expression in breast cancer and the role of trypsin on growth and metabolism of breast cancer cell line MDA MB-231.
29. Lin ZM, Zhao JX, Duan XN, Zhang LB, Ye JM, Xu L, Liu YH: Effects of tissue factor, PAR-2 and MMP-9 expression on human breast cancer cell line MCF-7 invasion. *Asian Pacific journal of cancer prevention : APJCP* 2014, 15(2):643-646.

30. Arisawa T, Tahara T, Shibata T, Nagasaka M, Nakamura M, Kamiya Y, Fujita H, Takagi T, Hasegawa S, Wang FY et al: Promoter hypomethylation of protease-activated receptor 2 associated with carcinogenesis in the stomach. *Journal of gastroenterology and hepatology* 2007, 22(6):943-948.

31. Guo D, Zhou H, Wu Y, Zhou F, Xu G, Wen H, Zhang X: Involvement of ERK1/2/NF-κB signal transduction pathway in TF/FVIIa/PAR2-induced proliferation and migration of colon cancer cell SW620. *Tumour biology : the journal of the International Society for Onco developmental Biology and Medicine* 2011, 32(5):921-930.

32. Wilson S, Greer B, Hooper J, Zijlstra A, Walker B, Quigley J, Hawthorne S: The membrane-anchored serine protease, TMPRSS2, activates PAR-2 in prostate cancer cells. *The Biochemical journal* 2005, 388(Pt 3):967-972.

33. Ikeda O, Egami H, Ishiko T, Ishikawa S, Kamohara H, Hidaka H, Mita S, Ogawa M: Expression of proteinase-activated receptor-2 in human pancreatic cancer: a possible relation to cancer invasion and induction of fibrosis. *International journal of oncology* 2003, 22(2):295-300.

34. Ikeda O, Egami H, Ishiko T, Ishikawa S, Kamohara H, Hidaka H, Takahashi M, Ogawa M: Signal of proteinase-activated receptor-2 contributes to highly malignant potential of human pancreatic cancer by up-regulation of interleukin-8 release. *International journal of oncology* 2006, 28(4):939-946.

35. Kothari A, Flick MJ: Coagulation Signaling through PAR1 as a Therapeutic Target in Pancreatic Ductal Adenocarcinoma. *Int J Mol Sci* 2021, 22(10).

36. Jiang P, Yu GY, Zhang Y, Xiang Y, Hua HR, Bian L, Wang CY, Lee WH, Zhang Y: Down-regulation of protease-activated receptor 4 in lung adenocarcinoma is associated with a more aggressive phenotype. *Asian Pacific journal of cancer prevention : APJCP* 2013, 14(6):3793-3798.

37. Zhang H, Jiang P, Zhang C, Lee S, Wang W, Zou H: PAR4 overexpression promotes colorectal cancer cell proliferation and migration. *Oncology letters* 2018, 16(5):5745-5752.

38. Rullier A, Senant N, Kisiel W, Bioulac-Sage P, Balabaud C, Le Bail B, Rosenbaum J: Expression of protease-activated receptors and tissue factor in human liver. *Virchows Archiv : an international journal of pathology* 2006, 448(1):46-51.

39. Lin YP, Nelson C, Kramer H, Parekh AB: The Allergen Der p3 from House Dust Mite Stimulates Store-Operated Ca(2+) Channels and Mast Cell Migration through PAR4 Receptors. *Molecular cell* 2018, 70(2):228-241.e225.
40. Gratio V, Walker F, Lehy T, Laburthe M, Darmoul D: **Aberrant expression of proteinase-activated receptor 4 promotes colon cancer cell proliferation through a persistent signaling that involves Src and ErbB-2 kinase.** *International journal of cancer* 2009, **124**(7):1517-1525.

**Figures**

![Figure 1](image-url)
The expression levels of PAR1 and PAR4 are inversely proportional in ESCC (A) PAR1 and PAR4 expression in ESCC tissue and normal tissue (para-carcinoma tissue) detected by immunohistochemistry, representative photomicrographs of immunohistochemical staining was shown in (B). (C) Western blot analysis and (D) RT-PCR analysis of expression of PAR1 and PAR4 in protein level and mRNA level, respectively. (E) PAR1 and PAR4 mRNA expression in esophageal patience and normal person based on TCGA database. (F) PAR1 and PAR4 expression in HEEC cells and ESCC cell lines EC109, Kyse140, Kyse150 and TE-1. Each bar represents the mean ± SD of three independent experiments; *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 2

PAR1 exerts an oncogene, while PAR4 exerts a tumor suppressor in ESCC (A). PAR1 and PAR4 expression in ESCC tissue and normal tissue (para-carcinoma tissue) detected by immunohistochemistry, representative photomicrographs of immunohistochemical staining were shown in (B). (C) Western blot analysis of PAR1 and PAR4 expression in protein level. (D) Kaplan-Meier analysis of Disease Free Survival in ESCC patients according to the data from the TCGA.
database. The log-rank test was used to calculate the DFS rate for comparison between different groups. (E) Western Blot analysis of PAR1 and PAR4 expression in HEEC, Kyse140 and TE-1 cells with respective siRNA transfection. (F, G) The effect of PAR1 or PAR4 KD on Kyse140 proliferation was detected by MTT assay. Cell viability was calculated in 24, 48, 72 and 96 hrs respectively. (H) Wound-healing assay showing the migration ability of Kyse140 cells transfected with PAR1 or PAR4 shRNA. (G) Transwell assay showing the migration ability of Kyse140 cells with indicated pre-treatment. These tumor cells which passed through the Transwell membranes were dissolved in methanol and quantified by microplate reader OD 405 nm. Result was shown in (J). Each bar represents the mean ± SD of three independent experiments; Scale bar, 200μm in wound healing assay, 100μm in Transwell assay; *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 3

PAR1 promoted the growth and metastasis of ESCC cell line, while PAR4 had an inhibitory effect in vitro (A) Effects of PAR1/PAR4 on intracellular calcium mobilization in Kyse140 cell. (B) The effect of PAR1 AP or PAR4 AP on Kyse140 proliferation was detected by MTT assay. Cell viability was calculated in 24, 48, 72 and 96h respectively. (C) The FACS analyses of PAR1 AP and PAR4 AP induced tumor cell apoptosis. Percentage of apoptotic cells compared to control was quantitated by mean fluorescence intensity and cell viability. (D) Western blot analysis of apoptosis-related proteins Bcl-2, c-Casp3, c-Casp9 and β-actin. Loading control was quantitated by mean fluorescence intensity and cell viability. (E) Representative images of cell morphology at 0h and 24h in WT, WT + PAR1 AP, WT + PAR4 AP conditions. (F) Representative images of migration assay conducted in PAR1 AP, WT and PAR4 AP conditions. (G) Bar graph showing mean migration rate ± SEM for PAR1 AP, NC and PAR4 AP conditions. 

PAR1 AP PAR4 AP PAR1 AP

| Protein   | NC | PAR4 AP | PAR1 AP |
|-----------|----|---------|---------|
| Bcl-2     | 1  | 1.43    | 0.09    |
| c-Casp3   | 1  | 0.76    | 1.03    |
| c-Casp9   | 1  | 0.65    | 1.09    |
| β-actin   | 1  | 1       | 1       |
was shown in below graphs. (D) Western blot analysis of tumor apoptosis in Kyse140 tumor cell line with antibodies directed against apoptotic protein. β-actin was used as a loading control. (E) Wound-healing assay showing the migration ability of Kyse140 cells treated with PAR1 AP or PAR4 AP. (F) Transwell assay showing the migration ability of Kyse140 cells with indicated pre-treatment. These tumor cells which passed through the Transwell membranes were dissolved in methanol and quantified by microplate reader OD 405 nm. Result was shown in (G). Each bar represents the mean ± SD of three independent experiments; Scale bar, 200μm in wound healing assay, 100μm in Transwell assay; *P < 0.05; **P < 0.01; ***P < 0.001.
PAR1 promoted the proliferation and metastasis of ESCC cell line, while PAR4 had an inhibitory effect in vivo (A, D) Western Blot analysis of PAR1 and PAR4 expression in TE-1 cells after knockdown or overexpression. (B, C) PAR1 overexpression, PAR1 knockdown and NC TE-1 cells were injected subcutaneously. Tumor volume was monitored within 50 days (B), and the tumor was dissected and weighted after 50 days (C). (E, F) PAR4 overexpression, PAR4 knockdown and NC TE-1 cells were injected subcutaneously. Tumor volume was monitored within 50 days (E), and the tumor was dissected and weighted after 50 days (F). (G, I) Pulmonary metastasis was assessed after TE-1 cells injection through the lateral tail vein. Metastasis was analyzed 50 days after injection of tumor cells. Average number of lung metastasis in each of the groups was showed. (H, J) Representative histologic evidence from tumor sections of the different groups. Four percent of paraformaldehyde-embedded lungs of all mice were cut completely, stained with hematoxylin and eosin, examined histologically and detected by microscopy. Each bar represents the mean ± SD of three independent experiments; *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 5

The co-activation of STAT3 and NF-κB is involved in the PAR1 activation-induced tumor promoting effect, while only NF-κB participates in the PAR4 activation-induced tumor inhibitory effect in ESCC (A, C, E) Kyse140 cell was treated with indicated way. Western blot analyzed the expression of p-STAT3/p-NF-κB in nucleoprotein and expression of STAT3/NF-κB in total protein level of Kyse140 cell. (B, D, F) Confocal microscopy and immunofluorescence staining to detect p-STAT3/STAT3/p-NF-κB expression in Kyse140 cell. (G) Western blot analyzed the expression of p-STAT3/p-NF-κB in nucleoprotein and expression of STAT3/NF-κB in total protein level of Kyse140 cell.
cells. (G) The effect of STAT3 inhibitor on Kyse140 proliferation was detected by MTT assay. (H) Pulmonary metastasis was assessed after Kyse140 cells injection through the lateral tail vein. Metastasis was analyzed 50 days after injection of tumor cells. Average number of lung metastasis in each of the groups was showed. Each bar represents the mean ± SD of three independent experiments; *P < 0.05; **P < 0.01; ***P < 0.001.
nSMase2/MAPK/NF-κB mediated PAR4 activation-induced tumor inhibitory effect, and FAK/PI3K/AKT/STAT3/NF-κB mediated PAR1 activation-induced tumor promoting effect in ESCC (A) Kyse140 cells were lysed and immunoprecipitated with antibodies against control IgG, PAR1 or PAR4. Thereafter, they were immunoblotted with antibodies against nSMase2, PAR1 and PAR4. (B) Kyse140 cell was treated with indicated way. Western blot analysis of the expression of p-p38 MAPK. (C) Kyse140 cells were pre-incubated with 3-OMS (30uM), followed by the addition of either PAR1-AP or PAR4 AP to trigger the phosphorylation of IKKβ. The IKKβ phosphorylation was detected by FCM. (D) Kyse140 cells were pre-treated with PAR1 AP or PAR4 AP. FAK phosphorylation was detected by FCM. (E) Kyse140 cell was treated with indicated way. Western blot analysis of the expression of PI3K, p-Akt and Akt. (F) The effect of nSMase inhibitor on Kyse140 proliferation was detected by MTT assay. (G) Western blot analysis of silence efficiency of Akt knockdown. (H) Confocal microscopy and immunofluorescence staining to detect p-STAT3/p-NF-κB expression in NC cells and Akt KD Kyse140 cells. Each bar represents the mean ± SD of three independent experiments; *P < 0.05; **P < 0.01; ***P < 0.001.

**Figure 7**

The schematic illustration of the mechanism of PAR1- and PAR4- induced tumor promoting/inhibitory effects in ESCC
Supplementary Files

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