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Short communication

# Decreased expression of protease-activated receptor 4 in human gastric cancer

Yong Zhang[a,1], Guoyu Yu[a,b,1], Ping Jiang[a,c], Yang Xiang[a], Wenliang Li[d], Wenhui Lee[a], Yun Zhang[a,*]

[a] Key Laboratory of Animal Models and Human Disease Mechanisms, Kunming Institute of Zoology, Chinese Academy of Sciences, 32 East Jiao Chang Road, Kunming, Yunnan 650223, China
[b] Department of Biochemistry, Kunming Medical College, Kunming, Yunnan 650032, China
[c] Department of Pathology, Kunming Medical College, Kunming, Yunnan 650032, China
[d] Department of Gastroenterology, the First Affiliated Hospital of Kunming Medical College, Kunming 650032, China

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ABSTRACT

Protease-activated receptors (PARs) are a unique family of G-protein coupled receptors. PAR4, the most recently identified PAR member, was reported to be overexpressed during the progression of colon and prostate cancers. Though PAR4 mRNA was detected in normal stomach, the role of PAR4 in gastric cancer has not been investigated. In this study, differential expression of PAR4 was measured by real-time PCR ($n = 28$) and tissue microarrays ($n = 74$). We showed that PAR4 was located from basal to middle portions of normal gastric mucosa. PAR4 expression was remarkably decreased in gastric cancer tissues as compared with matched noncancerous tissues, especially in positive lymph node or low differentiation cancers. Furthermore, methylation of the PAR4 promoter in cell lines was assessed by treatment with 5-aza-2′-deoxycytidine and genomic bisulfite sequencing. AGS and N87 human gastric cancer cell lines did not express PAR4, as compared to HT-29 human colon cancer cell line with significant PAR4 expression. Treatment with 5-aza-2′-deoxycytidine restored PAR4 expression in AGS and N87 cells, which exhibited significantly more 5-methylcytosines in the PAR4 promoter compared with HT-29 cells. Our results revealed that down-regulation of PAR4 expression occurs frequently in gastric cancers and exhibits association with more aggressive gastric cancer. Interestingly, the loss of PAR4 expression in gastric cancers may result from hypermethylation of the PAR4 promoter.



## 1. Introduction

Protease-activated receptors (PARs) are a unique family of G-protein coupled receptors (GPCRs) (Coughlin, 2000). Four members have been cloned in human, namely, PAR1 through PAR4, sharing the common mechanism of activation by proteolysis (Coughlin, 2000; Ossovskaya and Bunnett, 2004). A wide range of proteases from the coagulation cascade, inflammatory cells and the digestive tract including activating proteases and disabling proteases cleave and activate/inhibit PARs with diverse roles ranging from hemostasis to pain transmission (Ossovskaya and Bunnett, 2004; Wang and Reiser, 2003). PARs could serve as sensors for injury and generate appropriate responses in the pathogenesis of inflammation and wound repair (Kawabata et al., 2008; McDougall et al., 2009; Shpacovitch et al., 2007; Vergnolle, 2005). Recently, PARs are found to be overexpressed in several malignancies, and implicated in tumor growth and metastasis (Dorsam and Gutkind, 2007). For instance, PAR1 is overexpressed in highly invasive breast cancer (Even-Ram et al., 1998), colon cancer (Darmoul et al., 2003), and advanced-stage prostate cancer (Daaka, 2004). And overexpression of PAR4 contributes to hepatocellular carcinoma cell migration (Kaufmann et al., 2007), formation of fibrotic response in the lung (Ando et al., 2007), and tumor growth of prostate and colon cancers (Black et al., 2007; Gratio et al., 2009). PAR4 is expressed in the stomach as verified by Northern blot (Xu et al., 1998). However, the role of PAR4 in gastric cancer has not been investigated.

Gastric cancer remains one of the leading causes of cancer-related deaths worldwide, and it is the third most common cancer in China, although its incidence has declined rapidly in some regions of the world (He et al., 2005; Jemal et al., 2009). The majority of gastric cancers are still diagnosed in advanced stages which is generally resistant to radiotherapeutic or chemotherapeutic treatment (Paoletti et al., 2010). Gastric cancer diagnosed at stage IV carried a poor prognosis with 5 year survival less than 5% (Ushijima and Sasako, 2004). However, if gastric cancer is diagnosed at an early stage, it is a curable disease. Therefore it is important to identify clinically useful biomarkers that can detect gastric cancer at an early stage (Anderson et al., 2010). Gastric cancer was believed to be caused by multifactor and developed in a multistep process. Many genetic alterations have been reported in gastric carcinogenesis, including overexpression of oncogenes, such as *c-erbB2* and

* Corresponding author. Tel.: +86 871 519 8515; fax: +86 871 519 8515.
*E-mail address:* zhangy@mail.kiz.ac.cn (Y. Zhang).
[1] These authors contributed equally to this work.

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*c-met* (Allgayer et al., 2000; Lee et al., 2000), as well as inactivation of tumor suppressor genes such as *p53*, *β-catenin* and *E-cadherin* (Becker et al., 1994; Ebert et al., 2003; Shiao et al., 1994), but it is still not sufficient to understand molecular mechanism underlying the progression of gastric cancer.

In this study, we showed the expression of PAR4 is reduced in gastric cancer, and the down-regulation of PAR4 was associated with the clinically aggressive phenotype.

## 2. Materials and methods

### 2.1. Patients and tissue samples

Ethical approval for this study was obtained by the Ethical Committee of Kunming Institute of Zoology, the Chinese Academy of Sciences. Tissues were taken from the tumor and a tumor-free location that was at least 6 cm from the tumor in the First Affiliated Hospital of Kunming Medical College. The non-neoplastic tissue was confirmed to be without any tumor cell infiltration by histological assessment. Immediately after removal, all tissues for molecular analysis were put in liquid nitrogen and stored at −80 °C until use. Gastric cancer tissue microarray representing 91 gastric cancers with their non-neoplastic resection margins constructed according (Kononen et al., 1998) were from Shanghai Outdo Biochip Center (Shanghai, China).

### 2.2. Cell culture

AGS and N87 human gastric cancer cells and HT-29 human colon cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA). AGS cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 containing 10% fetal calf serum (FCS) and supplied with 100 U/ml penicillin, and 100 mg/ml streptomycin. N87 and HT-29 cells were cultured in RPMI-1640 and DMEM media, respectively. The cells were grown in a humidified atmosphere with 5% $CO_2$ at 37 °C. For treatment of cells with 5-aza-2′-deoxycytidine (5-Aza-dC; Sigma, St. Louis, MO, USA), cells were seeded at a density of $1 \times 10^6$ cells in 60-mm dish. After 24 h, cells were treated with 10 μM of 5-Aza-dC. DMSO was treated in parallel as a control. Total cells were collected 3 days after addition of 5-Aza-dC and subjected to RT-PCR analysis.

### 2.3. RNA extraction and polymerase chain reaction (PCR)

RNA extraction and the first-strand cDNA synthesis were performed as previously described (Liu et al., 2008). For semi-quantitative reverse-transcribed PCR (RT-PCR), the primers (forward and reverse) used were as follows: PAR4 (244 bp product): 5′-GGCAACCTCTATGGTGCCTA-3′ and 5′-TTCGACCCAGTACAGCCTTC-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (321 bp product): 5′-TCGGAGTCAACGGATTTGGTCGTA-3′ and 5′-AGCCTTCTCCATGGTGGTGAAGA-3′. Amplicons were separated by electrophoresis in 2% agarose gel, stained with ethidium bromide and viewed under ultraviolet illumination. The identity of the PCR product was confirmed by DNA sequencing.

Quantitative real-time PCR was performed with a continuous fluorescence detector (Opticon Monitor, Bio-Rad, Hercules, CA, USA). PCR reaction was carried out using an SYBR Green real-time PCR kit (TaKaRa, Dalian, China) with the condition as the following: initial denaturation at 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s. The primers used for amplifying human PAR4 (147 bp) were 5′-CCTTCATCTACTACTACTACGTGTCG-3′ (forward) and 5′-ACTGGAGCAAAGAGGAGTGG-3′ (reverse). Amplification of GAPDH (107 bp), with the primers 5′-ATGGGGAAGGTGAAGGTCG-3′ (forward) and 5′-GGGGTCATTGATGGCAACAATA-3′ (reverse), was examined in parallel as an internal control. The identity of the PCR products was confirmed by DNA sequencing. Each sample was run three times. No template controls (no cDNA in PCR) were run to detect unspecific or genomic amplification and primer dimerization. Fluorescence curve analysis was carried using Opticon Monitor software. Relative quantitative evaluation of PAR4 levels were performed by E-method and expressed as a ratio of the transcript of PAR4 to GAPDH in the tumor tissue divided by a similar ratio in the non-neoplastic tissue of the same patient.

### 2.4. Tissue immunohistochemistry

Tissue immunohistochemistry was performed as described (Gratio et al., 2009). Briefly, antigen retrieval was performed by heating in an autoclave at 121 °C for 5 min. Dewaxed sections were pre-incubated with blocking serum and then incubated overnight with the anti-human PAR4 antibody (C-20, 1:1200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C. Specific binding was detected by a streptavidin–biotin-peroxidase assay kit (Maxim, Fujian, China). The section was counterstained with Harris hematoxylin. Direct microscopic micrographs were captured using a Leica DFC320 camera controlled by Leica IM50 software (Leica, Germany). Sections incubated with normal goat IgG were served as a negative control and the negative immunohistochemical control was devoid of any detectable immunolabeling. Specificity of the antibodies for PAR4 was confirmed by pre-incubation overnight at 4 °C with its respective antigen (Santa Cruz) in a 20-fold molar excess of antigen to antibody. Pre-incubation with PAR4 antigen resulted in an absence of immunolabeling.

Immunohistochemical staining was assessed semi-quantitatively by measuring both the intensity of the staining (0, 1, 2, or 3) and extent of staining (0, 0%; 1, 0–10%; 2, 10–50%; 3, 50–100%). The scores for the intensity and extent of staining were multiplied to give a weighted score for each case (maximum possible, 9). For the statistical analysis, the weighted scores were grouped into two categories where scores of 0–3 were considered negative and 4–9 positive (Moss et al., 2008).

### 2.5. Western blotting

Tissue samples were homogenized in radioimmunoprecipitation assay buffer (Sigma) with protease inhibitors cocktail (Sigma). The protein concentration was determined by a protein assay kit (Bio-Rad). Samples (containing 50 μg of protein) were loaded on an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then electro-transferred onto a PVDF membrane. The membrane was subsequently blocked with 3% bovine serum albumin (BSA) and incubated with anti-PAR4 antibody (C-20, 1:3500, 4 °C, overnight) and secondary antibody. Protein bands were visualized with Super Signal reagents (Pierce, Rockford, IL, USA). Specificity of the anti-PAR4 antibody was confirmed by pre-incubation with its antigenic peptide in a 20-fold molar excess of antigen to antibody. Pre-incubation with PAR4 antigen resulted in an absence of PAR4 band.

### 2.6. Bisulfite sequencing

Genomic DNA from cell lines was isolated with the Universal Genomic DNA Extraction Kit (TaKaRa) and bisulfite-converted using the Clontech EpiXplore™ Methyl Detection Kit (TaKaRa). PAR4 promoter sequences were amplified from bisulfite-converted DNA by PCR, purified from agarose gels and subcloned into the pBackZero T Vector (TaKaRa). For each sample, 14 individual clones were sequenced to identify methylated cytosine residues. PCR primer sequences (forward and reverse) were 5′-TTTAAGGGTGATTTTAGGAAAGGTTTAGAG-3′ and 5′-ACTATAACCTCAAACTTCCTACCTC-3′.