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Author(s)	JAHAN, Israt; FUJIMOTO, Jiro; ALAM, Syed Mahfuzul; SATO, Eriko; SAKAGUCHI, Hideki; TAMAYA, Teruhiko
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Expression of protease activated receptor-2 related to angiogenesis in tumor advancement of uterine endometrial cancers

ISRAT JAHAN, JIRO FUJIMOTO, SYED MAHFUZUL ALAM, ERIKO SATO, HIDEKI SAKAGUCHI and TERUHIKO TAMAYA

Department of Obstetrics and Gynecology, Gifu University School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan

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Abstract. Protease activated receptor-2 (PAR-2) is the second member of a novel family of G-protein coupled seven-transmembrane domain receptors. PAR-2 has been reported to be expressed in various tumors and play a vital role in the regulation of cancer cell growth. The purpose of this study was to clarify the roles of PAR-2 in the angiogenic pathway in uterine endometrial cancers. PAR-2 expression was analyzed in 61 uterine endometrial cancer and 15 normal endometrium tissue specimens. PAR-2 histoscores and mRNA levels were determined by immunohistochemistry and real-time RT-PCR, respectively. Microvessel counts were determined by immunohistochemistry for CD31 and factor VIII-related antigen. The localization of PAR-2 was dominant in the cancer cells of endometrial cancer tissues of all cases studied. PAR-2 histoscores highly correlated with PAR-2 mRNA levels in the same tissues ($r=0.87$, $p<0.001$). PAR-2 histoscores and mRNA levels both significantly increased in uterine endometrial cancers with clinical stages (I< II< III, $p<0.001$), dedifferentiation (G1< G2< G3, $p<0.001$) and myometrial invasion (A< B, $p<0.001$; B< C, $p<0.05$) in comparison to normal endometria. There were significant correlations between PAR-2 histoscores and mRNA levels with microvessel counts in uterine endometrial cancers. PAR-2 was upregulated during uterine endometrial cancer progression with dedifferentiation and myometrial invasion. Therefore, PAR-2 might work on tumor advancement of uterine endometrial cancers via angiogenic activity.

Introduction

Protease activated receptors (PARs), a family of four seven-transmembrane G-protein coupled receptors, are activated by serine proteases (1). These proteases cleave within the extracellular amino terminus to expose a tethered ligand domain that binds to and activates the receptors to initiate multiple signaling cascades. Proteases and PARs are responsible for disease and are targets for therapies. Proteases that activate PARs are generated during tissue damage and PARs regulate many biological processes that are critical in disease, including trauma, hemostasis, cell survival, inflammation and tumor formation. The microenvironment of tumors is replete with proteases, and tumor cells themselves express PARs (2).

The second member of the PAR family, PAR-2, is activated mainly by trypsin-like proteases (1). The gene encoding human PAR-2 was isolated from a human genomic cDNA library using hybridization to a probe derived from the 3' exon of the mouse PAR-2 gene (3) and subsequently cloned from human kidney cDNA (3,4) that was localized to chromosome 5q (5). PAR-2 is expressed in the gastrointestinal tract, pancreas, kidney, liver, lung, vasculature, eye, prostate, ovary and uterus (4,6). PAR-2 is also found in various tumor cell lines: A549 (lung adenocarcinoma), SW480 (colon adenocarcinoma), DU 145 (prostate carcinoma), PC-3 (prostate adenocarcinoma), PANC-1 (pancreatic duct cell carcinoma) and MKN-1 (gastric carcinoma) (4). Various tumor cells secrete trypsin, which can affect proliferation and mediate metastatic processes such as cellular invasion, extracellular matrix degradation, angiogenesis and tissue remodeling (2,7-9). In MKN-1 cells, trypsin stimulates an integrin $\alpha 5\beta 1$-dependent adhesion to fibronectin and proliferation through PAR-2 (9). In addition, PAR-2 plays an important role in promoting cell proliferation of colon cancer (10) and of pancreatic cancer (11,12). PAR-2 expression has also been observed in breast carcinoma, gastric carcinoma, lung adenocarcinoma, hepatocarcinoma, thyroid carcinoma, and ovarian carcinoma, where it initiates a cellular response to tissue damage incurred through the processes of cell metastasis (13). To investigate the role of PAR-2 in uterine endometrial cancer, we analyzed the immunohistochemical localization and mRNA expression of PAR-2 in uterine endometrial cancer tissues according to clinical backgrounds.

Materials and methods

Patients and tissues. Prior informed consent for the following studies was obtained from all patients and approval was given

Correspondence to: Dr Jiro Fujimoto, Department of Obstetrics and Gynecology, Gifu University School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan
E-mail: jf@cc.gifu-u.ac.jp

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Table I. Clinical background of uterine endometrial cancer patients (n=61).

Clinical background	Clinical stage			Histological grade			Myometrial invasion		
	I	II	III	G1	G2	G3	A	B	C
Number of patients	29	16	16	25	21	15	16	20	25

Stage I is carcinoma confined to the uterine corpus; stage II involves the corpus and the cervix, but has not extended outside the uterus; stage III extends outside of the uterus but is confined to the true pelvis. G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma. A, tumor limited to the endometrium; B, invasion to less than half the myometrium; C, invasion to more than half the myometrium.

by the Research Committee for Human Subjects, Gifu University School of Medicine. Sixty-one patients ranging from 33 to 77 years of age underwent resection for uterine endometrial cancers (29 stage I cases, 16 stage II cases and 16 stage III cases; and 25 well-differentiated, 21 moderately differentiated and 15 poorly differentiated endometrioid adenocarcinoma cases) as shown in Table I and 15 patients ranging from 35 to 46 years of age underwent hysterectomy for uterine leimyoma with a regular menstrual cycle with histologically normal endometrium. None of the patients had received any pre-operative therapy. The tissues of uterine endometrial cancer and uterine leiomyoma were obtained immediately after surgery. The tissues for RNA isolation were snap-frozen and stored at -80˚C, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin wax. The clinical staging of uterine endometrial cancers was determined by International Federation of Gynecology and Obstetrics (FIGO) classification (14).

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue samples (4-μm sections) from uterine endometrial cancers were cut with a microtome and dried overnight at 37˚C on a silanized-slide (Dako, Carpinteria, CA, USA). The protocol of universal Dako labelled streptavidin-biotin kit was followed for each sample. Samples were deparaffinized in xylene at room temperature for 30 min, rehydrated with graded ethanol and washed in phosphate buffer saline (PBS). The samples were then placed in 10 mM citrate buffer (pH 6.0) and boiled in a microwave for 10 min for epitope retrieval. Endogenous peroxidase activity was quenched by incubating tissue sections in 3% H_2O_2 for 10 min. The primary antibodies were goat PAR-2 (C-17, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse CD31 (Dako, Glostrup, Denmark) and rabbit factor VIII-related antigen (Zymed Laboratories, South San Francisco, CA, USA) and were used overnight at 4˚C at dilutions of 1:100, 1:10 and 1:2, respectively. The slides were washed and biotinylated secondary antibody (Dako) was applied for 30 min. After rinsing in PBS, streptavidin-conjugated horseradish peroxidase (Dako) was added for 30 min. Slides were then washed and treated with the chromogen 3, 3'-diaminobenzidine (Dako) for 5 min, then rinsed in PBS, and counterstained with Mayer's hematoxylin, dehydrated in graded ethanols, cleared in xylene and cover-slipped with a mounting medium, Entellan New (Merck, Darmstadt, Germany). For the negative controls of PAR-2, CD31 and factor VIII-related antigen, the corresponding pre-immune animal serums (goat, mouse and rabbit, respectively) (Dako) were used instead of the primary antibodies.

Assessment of histochemical score (histoscore). All sections of immunohistochemical staining for PAR-2 were evaluated in a semiquantitative fashion according to the method described by McCarty *et al* (15), which considers both the intensity and the percentage of cells stained at each intensity. Staining intensity was classified as 0 (none), 1 (weak), 2 (distinct), 3 (strong) and 4 (very strong). For each stained section, a value designated histoscore was obtained by application of the following algorithm: histoscore = $\Sigma(i+1)$xPi, where i and Pi represent intensity and percentage of cells that stain at each intensity, respectively, and corresponding histoscore was calculated separately.

Assessment of microvessel density (MVD). The MVD was assessed in sequential tissue sections stained with mouse CD31 and rabbit factor VIII-related antigen antibodies. Blood vessels with a clearly defined lumen or a well defined linear vessel shape, but not single endothelial cells, were taken into account for microvessel counting (16). Five areas of highest vascular density were chosen and microvessel counting was performed at high-power fields (x200) by two investigators. The microvessel counts (MVCs) were determined as the mean of the vessel counts obtained from these fields (17).

Preparation of standard template for real-time polymerase chain reaction (PCR). Internal standard template for real-time PCR was produced by PCR amplification using the primers of PAR-2 gene, 511-947 in the cDNA (PAR-2-TS: 5'-CTCC TCTCTGTCATCTGGTT-3' and PAR-2-TAS: 5'-CTGATC ATCAGCACATAGGC-3'). The DNA template was purified using a GeneClean II kit (Qbiogene, Irvine, CA, USA). The copy numbers of the standard template were determined to quantitate PAR-2 mRNA level in samples for real-time reverse transcription (RT) and PCR.

Real-time RT-PCR. Total RNA was extracted with the acid guanidinium thiocyanate-phenol-chloroform method (18). The total RNA (3 μg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (MMLV-RT, 200 U/μl, Invitrogen, Carlsbad, CA, USA) and the following

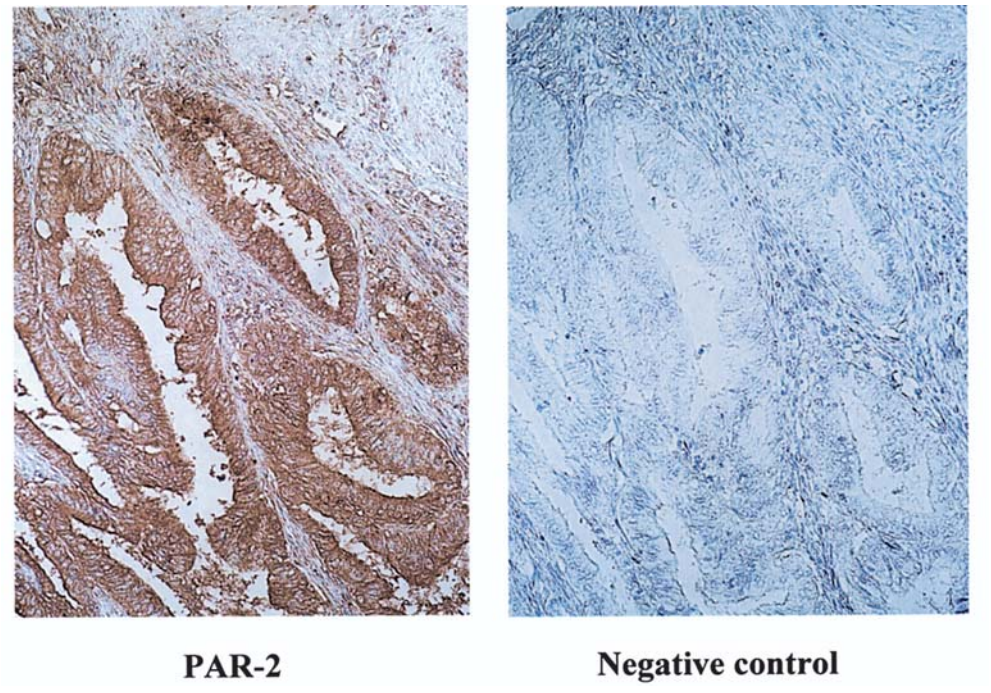

Figure 1. Immunohistochemical staining for PAR-2 in uterine endometrial cancers. A representative case of well-differentiated endometrioid adenocarcinoma of the endometrium. Goat anti-human PAR-2 antibody was used at a dilution of 1:100 as the primary antibody. Dark brown staining represents positive for PAR-2 antigen. Original magnification, x200.

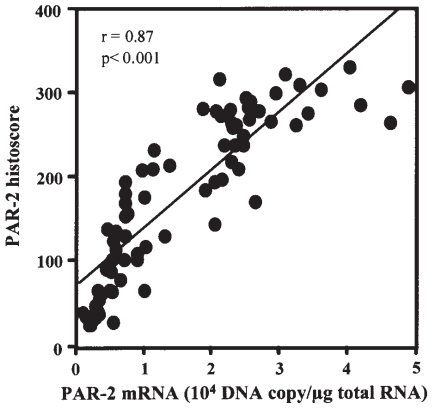

Figure 2. Correlation between PAR-2 histoscores and mRNA levels in normal endometria and uterine endometrial cancers. PAR-2 histoscores and mRNA levels were determined by immunohistochemistry and real-time RT-PCR, respectively. Each level is the mean ± SD of 9 determinations.

reagents: 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$, 0.1 M dithiothreitol, 10 mM deoxynucleotide (deoxyadenosine, deoxythymidine, deoxyguanosine and deoxycystidine) tri-phosphates (dNTPs) mixture and random hexamers (Invitrogen) at 37˚C for 1 h. The reaction mixture was heated for 5 min at 94˚C to inactivate MMLV-RTase.

Real-time PCR reaction was performed with a Takara Ex Taq R-PCR kit, version 1.0 (Takara, Otsu, Japan), using a smart cycler system (Cepheid, Sunnyvale, CA, USA). The reaction solution (25 μl) contained Takara Ex Taq HS (5 units/μl), 10X R-PCR buffer, 250 mM Mg^{++} solution, 10 mM dNTP mixture, SYBR Green I (1:1000 dilution; Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) and 20 μM of the primers of PAR-2 gene, 622-806 in the cDNA (PAR-2-S: 5'-AGAGGTATTGGGTCATCGTG-3' and PAR-2-AS: 5'-GC AGGAATGAAGATGGTCTG-3') with the transcribed total RNA from the tissue and a serially diluted standard template. The real-time PCR reactions were initially denatured by heating at 95˚C for 30 sec, followed by 40 cycles consisting of denaturation at 94˚C for 10 sec, annealing at 55˚C for 5 sec and extension at 72˚C for 20 sec. A strong linear relationship between the threshold cycle and the log concentration of the starting DNA copy number was always shown (correlation coefficient >0.99). Quantitative analysis was performed to determine the copy number of each sample.

Statistical analysis. PAR-2 mRNA levels were determined from three parts taken from each tumor, and each sample was analyzed in triplicate. The levels of PAR-2 were calculated using Student's t-test. The correlation coefficients were evaluated both by linear regression analysis and bivariate Pearson's correlation. Differences were considered significant when p-value was <0.05.

Results

PAR-2 localization by immunohistochemistry. PAR-2 was dominantly distributed in the cancer cells in all cases studied. Immunohistochemical staining for PAR-2 on a representative case of well-differentiated endometrioid adenocarcinoma of the uterine endometrium is shown in Fig. 1.

Correlation between PAR-2 histoscores and mRNA levels. PAR-2 histoscores highly correlated with PAR-2 mRNA levels in the same tissues, as determined by real-time RT-PCR ($r=0.87$, $p<0.001$), as shown in Fig. 2.

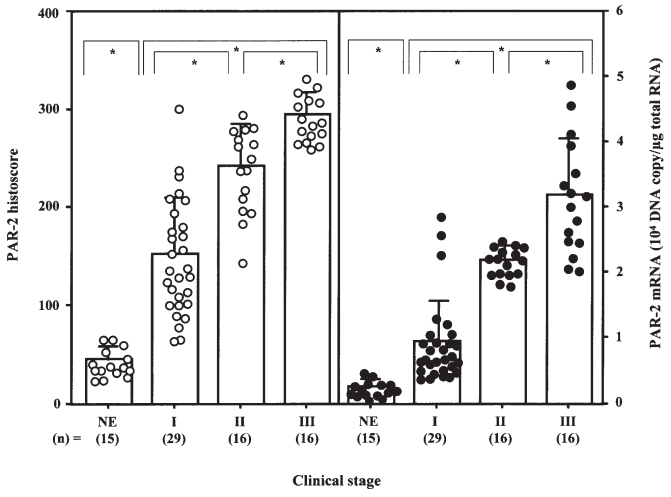

Figure 3. PAR-2 histoscores and mRNA levels in normal endometria and uterine endometrial cancers classified according to clinical stages. Clinical stages of uterine endometrial cancer were assessed according to FIGO classification. Each level is the mean ± SD of 9 determinations. NE, normal endometrium; *p<0.001.

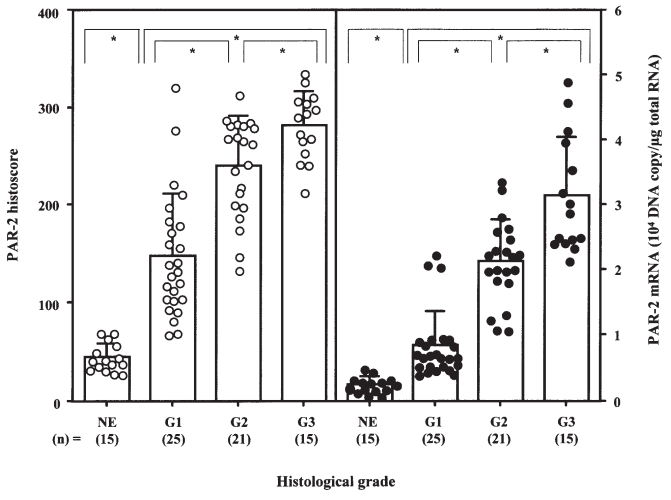

Figure 4. PAR-2 histoscores and mRNA levels in normal endometria and uterine endometrial cancers classified according to histological grades. Histological grades of uterine endometrial cancer were assessed according to FIGO classification. Each level is the mean ± SD of 9 determinations. G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma. NE, normal endometrium; *p<0.001.

PAR-2 histoscores analyzed by immunohistochemistry and PAR-2 mRNA levels analyzed by real-time RT-PCR. PAR-2 levels increased with the advancement of uterine endometrial cancers. PAR-2 histoscores and mRNA levels in uterine endometrial cancers both significantly increased with clinical stages (I< II< III, p<0.001) as shown in Fig. 3, with histological grades (G1< G2< G3, p<0.001) as shown in Fig. 4 and with depth of myometrial invasion (A< B, p<0.001; B< C, p<0.05) as shown in Fig. 5. Compared with normal endometria, the mean expression of the PAR-2 mRNA levels indicated 8.7 (p<0.001), 8.5 (p<0.001), 7.1 (p<0.001)-fold increase in the uterine endometrial cancers with clinical stages, histological

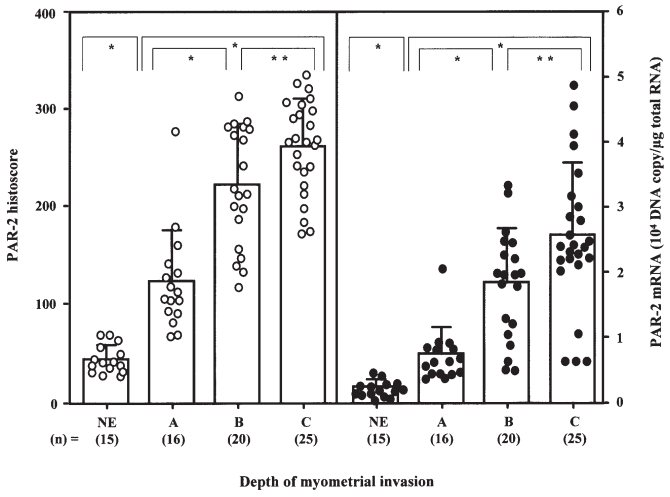

Figure 5. PAR-2 histoscores and mRNA levels in normal endometria and uterine endometrial cancers classified according to depth of myometrial invasion. Depths of myometrial invasion of uterine endometrial cancer were assessed according to FIGO classification. Each level is the mean ± SD of 9 determinations. A, tumor limited to the endometrium; B, invasion to less than half the myometrium; C, invasion to more than half the myometrium. NE, normal endometrium; *p<0.001; **p<0.05.

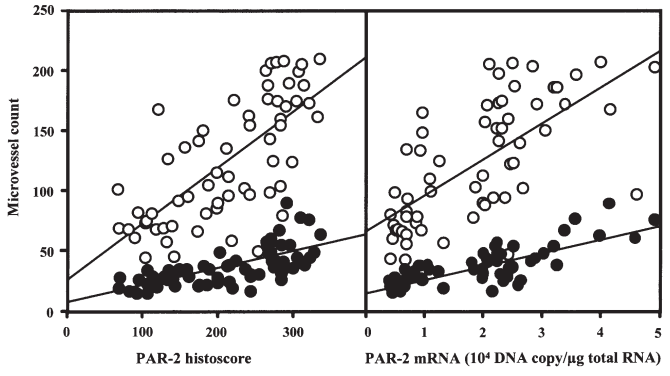

Figure 6. Correlation of microvessel counts (MVCs) with PAR-2 histoscores and mRNA levels in uterine endometrial cancers. White circles, MVCs by immunohistochemical staining for CD31; black circles, MVCs by immunohistochemical staining for factor VIII-related antigen.

grades and myometrial invasion, respectively (Figs. 3-5). Simultaneously, the mean expression of the PAR-2 histoscores in uterine endometrial cancers with clinical stages, histological grades and myometrial invasion also increased 5.2 (p<0.001), 5 (p<0.001), 4.6 (p<0.001)-fold compared to normal endometria, respectively (Figs. 3-5).

Association of PAR-2 histoscores and mRNA levels with microvessel counts. In immunohistochemistry, CD31 and factor VIII-related antigen (F-VIII) were clearly distributed in vascular endothelial cells. There were significant correlations between PAR-2 histoscores and MVCs by CD31 (r=0.70, p<0.001) and F-VIII (r=0.67, p<0.001) in uterine endometrial cancers, as well as between PAR-2 mRNA levels and MVCs by CD31 (r=0.68, p<0.001) and F-VIII (r=0.78, p<0.001), as shown in Fig. 6.

Discussion

Compared with normal endometrium PAR-2 was upregulated with increasing disease stage, dedifferentiation and myometrial invasion in the present study. PAR-2 is involved in cellular proliferation, invasion and metastasis with inflammation and angiogenesis (2,13,19-21). A synergistic effect of PAR-2 with VEGF in alveolar angiogenesis by proliferation of alveolar capillary endothelial cells has been observed in primary lung adenocarcinoma (22). PAR-2 mRNA expression is increased by 16-fold in pulmonary tumor alveolar walls, compared with in normal alveolar tissues (22). In breast tumor tissues, there is an upregulation of PAR-2 in proliferating stromal fibroblasts surrounding the carcinoma cells (13). PAR-2 mediates endothelial cell mitogenesis *in vitro* (23) and microvascular permeability *in vivo* (24), which are regarded as essential steps of the angiogenesis process.

That neovascularization is most pronounced in advanced stages suggests that an enhanced vascular supply reflects an increased malignant potential (25). Tumor cells rarely shed into the circulation before the primary tumor is vascularized (26). It has been shown that greater numbers of tumor vessels increase the opportunity for tumor cells to enter the circulation (27). Moreover, newly formed capillaries have fragmented basement membrane and are leaky, making them more penetrable by tumor cells than mature vessels (28). Furthermore, microvessel count is an independent significant prognostic factor in patients with breast cancer (29), and both relapse-free and overall survival rates decrease with increasing microvessel count (30). In the present study, positive correlation of PAR-2 expression with microvessel counts indicates that PAR-2 may be a candidate for angiogenic mediator as the clinical relevance of angiogenesis is assessed by MVD.

Specific angiogenic factors show specificity in the role of angiogenesis in each tumor's progression. The angiogenic factors vascular endothelial cell growth factor (VEGF), thymidine phosphorylase (TP) identified with platelet-derived endothelial cell growth factor (PD-ECGF), basic fibroblast growth factor (bFGF), interleukin-8, ETS-1 and cyclooxygenase-2 work on angiogenesis in uterine endometrial cancers (31-36). We previously reported that bFGF expression was upregulated with advancement and dedifferentiation (33) and conversely VEGF and TP expressions were downregulated with dedifferentiation (G1> G2> G3) and clinical stages (31,32). In this study, PAR-2 levels gradually increased with progression of uterine endometrial cancers and thus may be a good prognostic indicator in uterine endometrial cancers.

Angiogenic factors from tumors induce and activate matrix metalloprotease, plasminogen activator, collagenase and other enzymes in endothelial cells and thus facilitate the proliferation and migration of endothelial cells by dissolving the basement membrane and interstitial matrix protein. Basic FGF, expressed in cancer and stromal cells, works on basic angiogenesis (33). VEGF is the most sensitive angiogenic factor and is expressed in cancer cells. The VEGF isomers VEGF165 and VEGF121 rapidly move and bind to the receptors on endothelial cells (31). TP, expressed in interstitial cells, contributes to myometrial invasion in the early stages of uterine endometrial cancers (32). In the present findings, PAR-2 was localized in the cancer cells of uterine endometrial cancers. The interaction of endometrial cancer cells with endothelial cells initiated by PAR-2 might activate the process of angiogenesis.

In conclusion, potentiation of PAR-2 activation might induce angiogenesis in uterine endometrial cancer. Our study provides new insights into PAR-2 as a plausible novel angiogenic mediator in uterine endometrial cancer progression, and thus may be an attractive target for therapeutic approaches.

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