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ORIGINAL RESEARCH

Expression of protease activating receptor-2 (PAR-2) is positively correlated with the recurrence of non-muscle invasive bladder cancer: an immunohistochemical analysis

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Background: Matriptase, which is a Type II transmembrane serine protease, has the potential to activate several growth factors, including pro-hepatocyte growth factor (HGF). A G protein-coupled transmembrane cell-surface receptor and a protease-activated receptor 2 (PAR-2) are also required for activation by matriptase. Activation of PAR-2 has been reported to induce the progression of various cancers. In a previous study, we evaluated the correlation between upregulation of MET phosphorylation with high matriptase expression and worse prognosis in patients with muscle invasive bladder cancer; however, expression of PAR-2, matriptase and MET in non-muscle invasive bladder cancer (NMIBC) has not been evaluated.

Materials and methods: We retrospectively analyzed the expression of PAR-2, matriptase and MET using 55 paraffin-embedded specimens obtained from patients with NMIBC by immunohistochemistry.

Results: MET was significantly expressed in high-grade urothelial carcinoma (UC) and pathological T1 cancers. High expression of PAR-2 was significantly associated with a worse recurrence rate in NMIBC. In subgroup analysis, the expression of PAR-2 was also correlated with high recurrence rate in low-grade UC. In addition, expression of matriptase tended to correlate with worse recurrence rate in high-grade UC.

Conclusion: Increased expression of PAR-2 was significantly correlated with worse recurrence rate in patients with NMIBC. In addition, expression of matriptase also indicated a tendency toward recurrence in high-grade UC, suggesting an important role of matriptase-induced PAR-2 activation in NMIBC.

Keywords: PAR-2, matriptase, MET, NMIBC

Introduction

Bladder cancer is a common malignancy that affects elderly males worldwide. Approximately 70–80% of the detected tumors are classified as non-muscle invasive bladder cancer (NMIBC) at initial diagnosis.[1] NMIBC is generally managed by transurethral resection (TUR); however, a large number of patients have a 50–70% risk of recurrence after first TUR.[2,3] In addition, 15–25% of the patients are also at risk of progression to invasive cancer.[4] Therefore, the search for biomarkers of NMIBC recurrence and progression is important. Although a number of reports

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have discussed the prediction of recurrence, including clinicopathological parameters and biomarkers, critical predictive markers have yet to be identified.[4,5]

In a previous study, we evaluated the correlation between upregulation of MET phosphorylation and worse prognosis in patients with muscle invasive bladder cancer.[6] In addition, the population with increased expression of matriptase, which is a member of the Type II transmembrane serine protease family, also presented poor prognosis.[6] Matriptase has the potential to activate several growth factors, including pro-hepatocyte growth factor (HGF), pro-platelet-derived growth factor D, pro-macrophage stimulating protein-1 and protease-activated receptor 2 (PAR-2).[7]

PAR-2 is a G protein-coupled transmembrane cell-surface receptor which is activated by several serine proteases with tryptic specificity, including trypsin, coagulation factor VII/tissue factor complex, coagulation factor X, tryptase and matripatse.[8,9] Proteolytic activation of PAR-2 induces various responses in cancer cells such as increased cell proliferation, motility, invasiveness and hypoxia-induced angiogenesis.[8]

Expression of matriptase and PAR-2 in NMIBC have not been evaluated, and their functions have not yet been fully analyzed. In our current study, therefore, we analyzed the expression of matriptase, PAR-2 and MET, a well-known tyrosine kinase receptor of HGF, in NMIBC specimens immunohistochemistry.

Materials and methods

We conducted a retrospective study with data from clinical records and tumor specimens obtained from paraffin-embedded blocks. The experimental protocol was approved by the Ethical Review Committee of Miyazaki University. We examined a series of 55 specimens of bladder cancer collected by TUR at our hospital between 2010 and 2014. The patients whose tissues were used in this research provided written informed consent, in accordance with the Declaration of Helsinki. No patient received previous treatment. A single immediate postoperative intravesical instillation of pirarubicin was performed for all patients. Exclusion criteria included: 1) patients with a history of intravesical installation of Bacillus Calmette–Guerin; 2) patients with residual cancers after second-TUR for pT1 high-grade UC; 3) unsuitability of specimens for immunohistochemical analysis (such as severe heat degeneration). The bladder cancers were staged according to TNM classification, and pathological diagnosis was performed by two pathologists in accordance with the World Health Organization classification of tumors.[10]

Immunohistochemistry and analysis

Formalin-fixed paraffin-embedded sections were prepared according to routine method. Specimens were used for hematoxylin and eosin stain, and immunohistochemistry. Anti-human MET rabbit polyclonal antibody was purchased from Immuno-Biological Laboratories (Gunma, Japan) and anti-human ST14/matriptase polyclonal antibody was purchased from LifeSpan Biosciences (Seattle, WA, USA). Rabbit polyclonal anti-human PAR-2 antibody was purchased from Abcam (Cambridge, UK). For immunohistochemistry, sections were processed for antigen retrieval (microwaved in 10 mM citrate buffer, pH 6.0 for 10 mins), followed by treatment with 3% H_2O_2 in methanol for 10 mins and washed in phosphate-buffered saline (PBS) twice. After blocking in 3% bovine serum albumin and 5% goat serum in PBS for 2 hrs at room temperature, the sections were incubated with primary antibodies overnight at 4°C. Negative controls did not include the primary antibody. Sections were then washed in PBS and incubated with Envision-labeled polymer reagent (DAKO, Carpinteria, CA, USA) for 30 mins at room temperature. Sections were treated with nickel, cobalt-3, 3-diaminobenzidine (Immunopure Metal Enhanced DAB Substrate Kit; Pierce, Rockford, IL, USA) and counterstained with hematoxylin.

Immunoreaction staining intensity was judged by percentage of bladder cancer cells in which the cancer cell membranes were stained with or without staining of cytoplasm (eg, if 80 out of 100 cells were stained, staining was 80%): staining of >60%, strongly positive (2+); 20–60%, positive (1+); 5–20%, weakly positive, negative (−). Evaluation was performed by two experienced pathologists. We regarded a 2+ finding as high expression, and 1+ and – findings as low expression for all molecules.

Statistical analysis

Statistical parameters were assessed using SPSS statics, version 17.0 (SPSS, Chicago, IL, USA). For analysis of follow-up data, overall survival was calculated by Kaplan–Meier method; survival distributions were compared by log-rank test. Associations were determined by χ^2-test, and a P-value of <0.05 was set for statistical significance.

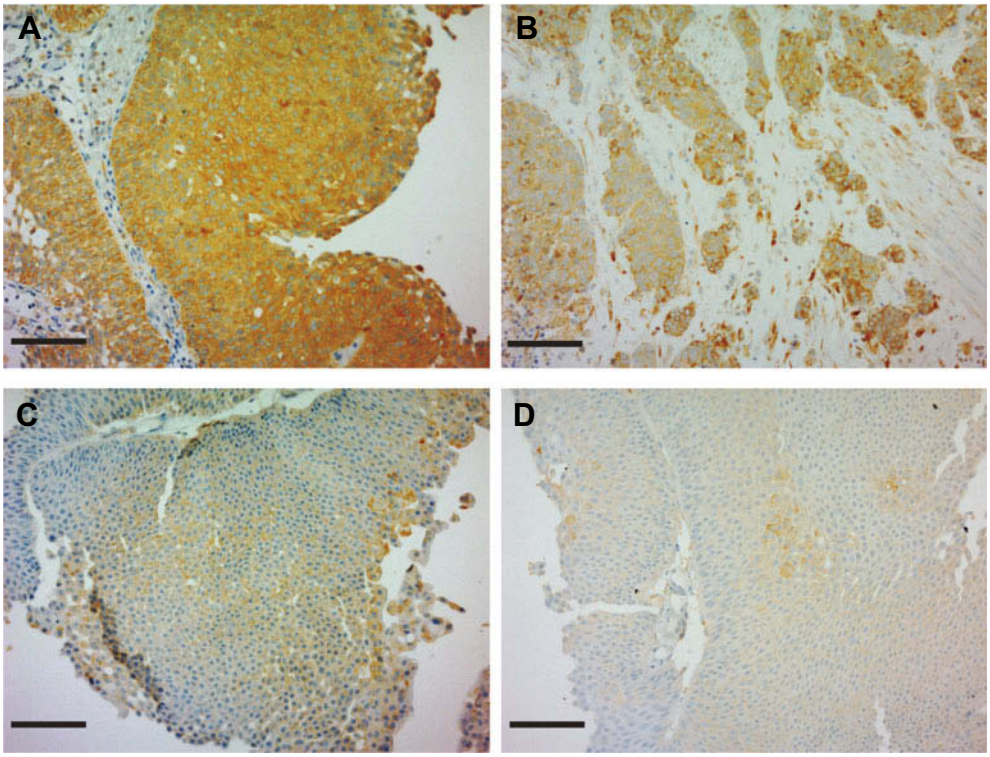

Figure 1 Representative result of PAR-2 immunoreactivity in non-muscle invasive bladder cancer (NMIBC). Tumor cells from 29 (53%) NMIBC specimens were stained strongly positive (**A** and **B**), which was regarded as high expression. Whereas, tumor cells from 26 (47%) specimens were stained positive, slightly positive (**C** and **D**) or negative, which was regarded as low expression. Scale bar =100 μm.

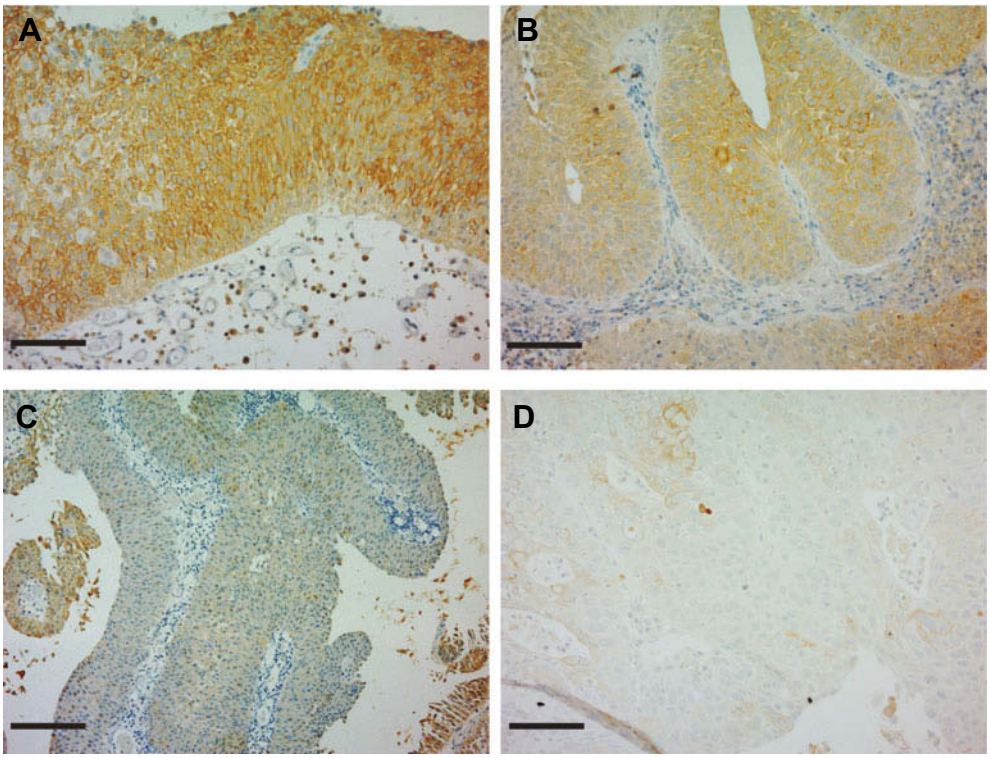

Figure 2 Representative result of matriptase immunoreactivity in non-muscle invasive bladder cancer (NMIBC). Tumor cells from 23 (42%) NMIBC specimens were strongly immunostained for matriptase (**A** and **B**), which was regarded as high expression. Whereas, tumor cells from 32 (58%) specimens were stained positive, slightly positive (**C** and **D**) or negative, which was regarded as low expression. Scale bar = 100 μm.

Results

Expression of PAR-2, matriptase and MET in NMIBC tissue

A total of 55 specimens were used for analysis, including 29 specimens of low-grade urothelial carcinoma (UC) and 26 specimens of high-grade UC. Immunohistochemical appearance is shown in Figures 1–3. Positive staining of all molecules, which was defined as membranous staining with or without cytoplasmic stain, was observed in cancer cells (Figures 1–3A and B). Negative staining is also shown (Figures 1–3C and D). As a result, high expression of PAR-2 was observed in 29 cases (53%). High expression of PAR-2 was observed in 15 cases (52%) of low-grade UC and 14 cases (54%) of high-grade UC. High expression of matriptase was observed in 23 cases (42%), and MET was observed in 11 cases (20%). Eleven cases (38%) of high matriptase expression were observed in low-grade UC and in 12 cases of high-grade UC. High expression of MET was observed in 2 cases (7%) of low-grade UC and 9 cases (37%) of high-grade UC.

Correlation between PAR-2, matriptase and MET expression and clinicopathological parameters

We analyzed the correlation between patient characteristics and the results of immunohistochemistry. As shown in Table 1, expression of MET was positively correlated with pathological grade (low-grade versus high grade: P=0.01029) and pathological T stage (pTa versus pT1: P=0.01324). Expression of PAR-2 was significantly increased in male patients (P=0.00834).

Next, we examined the correlation between PAR-2 and matriptase or MET. The results are shown in Table 2. No statistical correlation was observed between these molecules.

Recurrence-free survival according to PAR-2, matriptase and MET in patients with NMIBC

To evaluate the prognostic effect of matriptase, PAR-2 and MET for NMIBC, we analyzed recurrence-free survival using the Kaplan–Meier method. As a result, increased

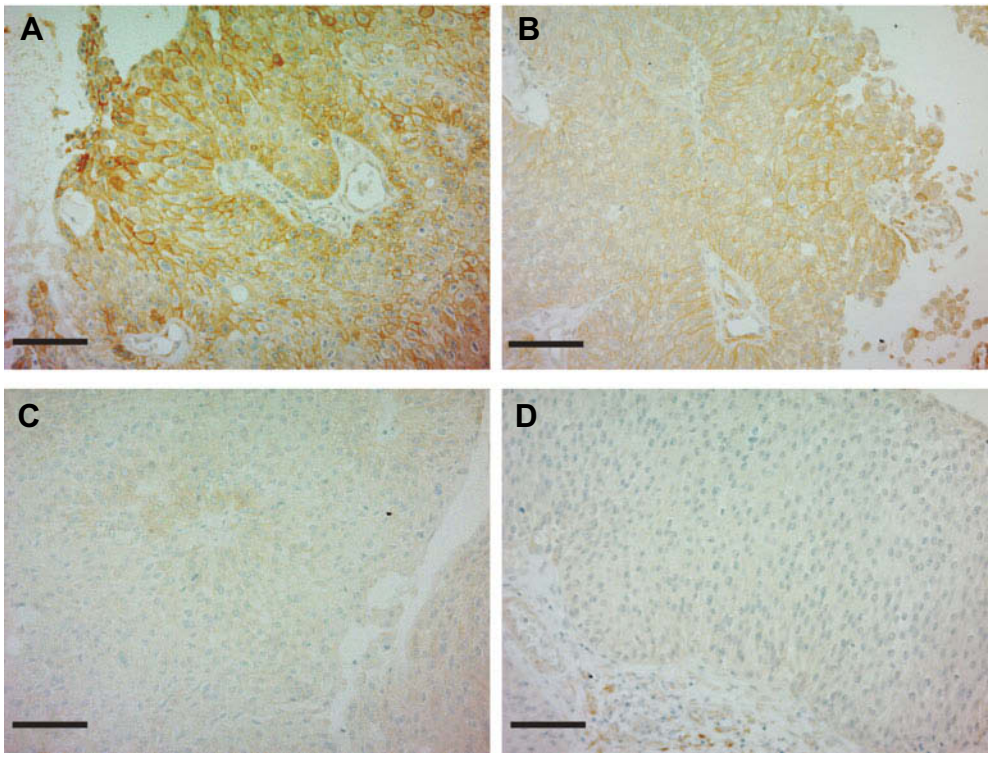

Figure 3 Representative result of MET immunoreactivity in non-muscle invasive bladder cancer (NMIBC). Tumor cells from 11 (20%) NMIBC specimens were stained strongly positive (**A** and **B**), which was regarded as high expression. Whereas, tumor cells from 44 (80%) specimens were stained positive, slightly positive (**C**) or negative (**D**), which was regarded as low expression. Scale bars=100 μm.

Table 1 Correlationbetween expression of PAR-2, MET, matriptaseand clinicopathological parameters

	PAR-2			Matriptase			MET		
	Low	High	*P*-value*	Low	High	*P*-value*	Low	High	*P*-value*
Mean age, years	69	72		68	74		71	70	
Gender									
Male	14	25	**0.00834***	22	17	0.67752	32	7	0.55267
Female	12	4		10	6		12	4	
Pathological grade									
Low	14	15	0.87495	18	11	0.53708	27	2	**0.01029***
High	12	14		14	12		17	9	
pT-stage									
Ta	16	17	0.82547	21	12	0.31518	30	3	**0.01324***
T1	10	12		11	11		14	8	

Note: *Statistical significant values ($P<0.05$) are indicated in bold.

Table 2 Correlation between PAR-2and MET, Matriptase

		MET			Matriptase		
		Low	High	*P*-value	Low	High	*P*-value
PAR-2	Low	21	5	0.89258	18	8	0.11573
	High	23	6		14	15	

PAR-2 expression was significantly correlated with high recurrence rates ($P=0.002$, Figure 4A). However, no relationship was observed between matriptase, MET and recurrence (Figure 4B and C). Next, we analyzed recurrence-free survival in patients with low-grade and high-grade UC. Increased expression of PAR-2 was also correlated with recurrence of low-grade UC (Figure 5A, $P=0.0327$); however, no significant correlation was observed in high-grade UC (Figure 5B). Although increased expression of matriptase indicated a tendency toward recurrence in patients with high-grade UC, statistical significance was not achieved ($P=0.1366$, Figure 5C). Because only two patients were positive for MET staining in patients with low-grade UC, we analyzed the recurrence rate for patients with high-grade UC only. However, no statistical correlation was observed.

Discussion

PAR-2 encoded gene (*F2RL1*) is reported to be located at human chromosome 5q 13.3.[11] The protein is composed of 397 amino acids with a molecular weight of 44 kDa.[11] On the cell surface, proteolytic cleavage of the specific site at N-terminus by the activators generates a new N-terminus motif that functions as a tethered ligand to activate PAR-2.[12,13] The function is mediated through the activation of several signaling pathways, including phospholipase C, intracellular calcium, mitogen-activated protein kinase, I-kappaB kinase/NF-kappaB and Rho.[14–16] PAR-2 is highly expressed in various cancers, and it is reported to promote hypoxia-induced angiogenesis, cell motility and invasive activities through its activation.[17] On the other hand, activation of PAR-2 has also been reported to promote cancer cell proliferation in gastric, colon, pancreatic and cervical cancer, and blocking of PAR-2 activity reported to suppress tumor growth in vivo.[16] With the exception of an in vitro study using RT4 cell line, however, the expression of PAR-2 in urothelial cancer has not been evaluated.[17] In our study, the expression of PAR-2 was positively correlated with high recurrence rate in NMIBC, especially in low-grade UC. To the best to our knowledge, this is the first report describing the expression of PAR-2 in NMIBC. The significance of matriptase-induced PAR-2 activation in proliferation or anti-apoptotic effect may be suggested;[18] however, additional examination is required to clarify the mechanism. In addition,

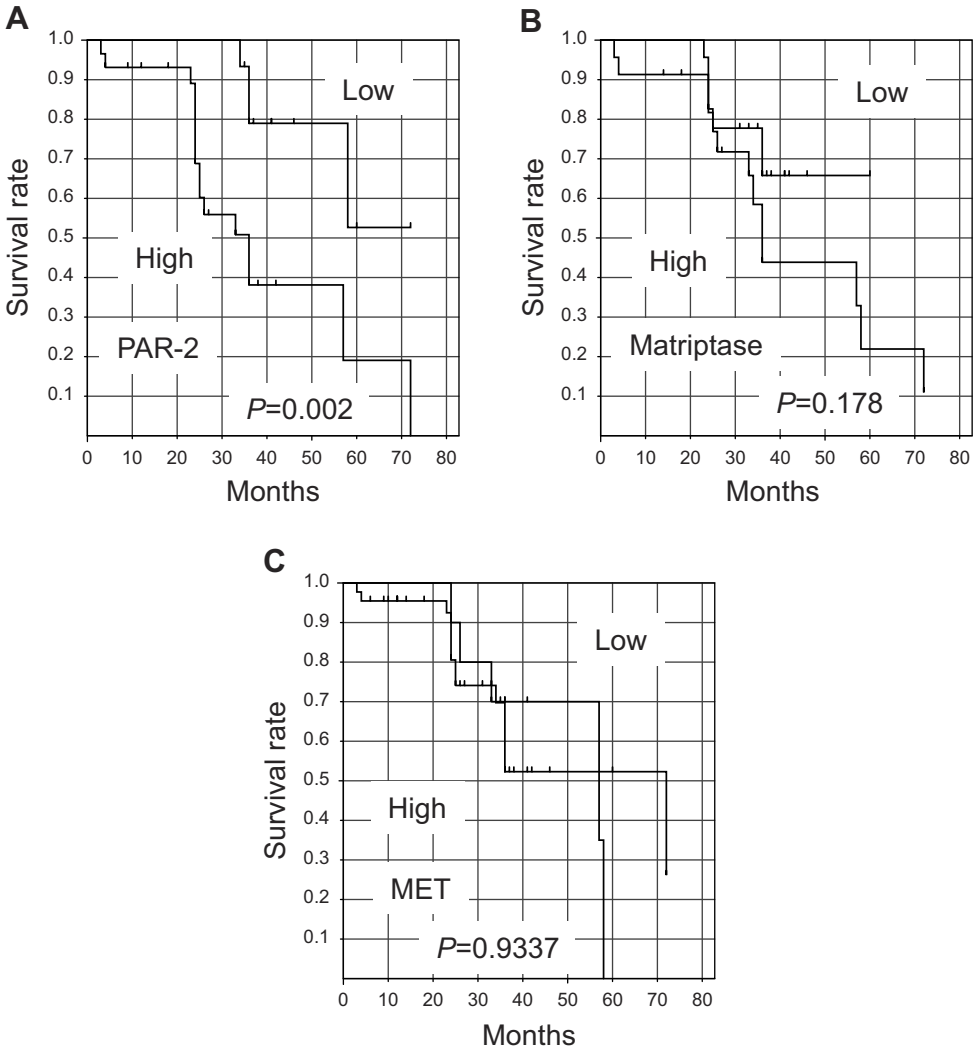

Figure 4 Comparison of recurrence-free survival (RFS) rates of non-muscle invasive bladder cancer patients in PAR-2 (**A**), matriptase (**B**) or MET (**C**) high expression and low expression groups. The RFS rate of the PAR-2-low group was significantly better than that of the PAR-2-high group. Expression of matriptase, MET and RFS were not associated.

similar analysis using a larger number of specimens are required to clarify the significance of PAR-2 expression in NMIBC.

HGF is a multifunctional growth factor known to play an important role in tumor progression via its specific receptor tyrosine kinase MET, the *c-met* proto-oncogene product.[19] HGF is secreted primarily by fibroblasts as an inactive single-chain precursor (pro-HGF/SF) that lacks biological activity and requires proteolytic cleavage to become the active, two-chain mature form.[7,20] As mentioned above, matriptase is the most efficient pericellular activator of pro-HGF. The matriptase gene (*ST14*) is located on human chromosome 11q24-25.[21] The gene encodes 855 amino acids, and the molecular weight of the protein is 80–90-kDa.[22] Matriptase is synthesized as an inactive, single chain zymogen, and activation requires two sequential endoproteolytic cleavages.[21] Matriptase is highly expressed in breast cancer cell lines, and it is involved in cancer progression through activation of the HGF/MET signaling axis.[23,24] Co-expression of matriptase and MET has also been reported in head and neck cancer, and renal cell carcinoma with poor prognosis.[24,25] Our study demonstrated increased expression of MET in high-grade, invasive (T1) UC, and increased expression of matriptase indicated a tendency toward recurrence in high-grade UC.

Conclusion

MET was significantly expressed in high-grade UC and T1 cancers. Increased expression of PAR-2 was significantly correlated with worse recurrence rate in patients with

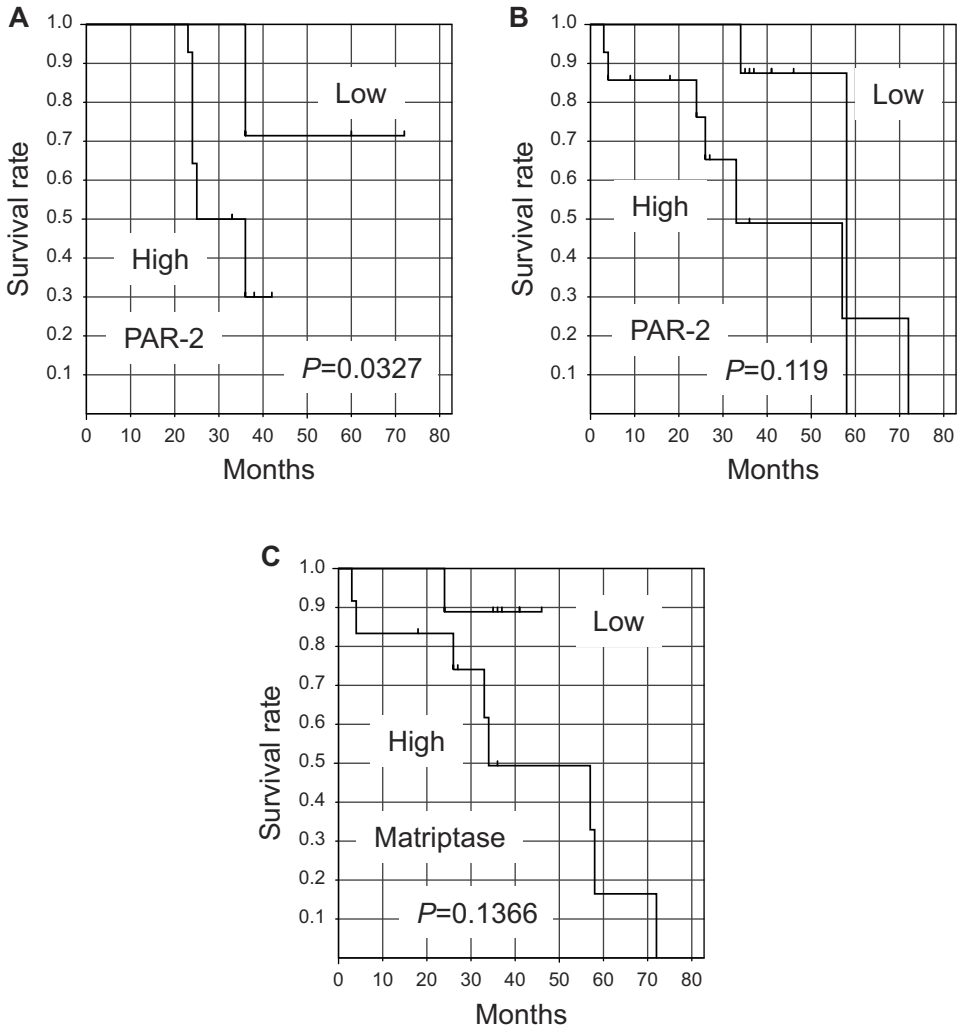

Figure 5 Comparison of recurrence-free survival (RFS) rates of low-grade urothelial carcinoma (UC) (**A**) and high-grade UC (**B**) in PAR-2-high groups. The RFS rate of the PAR-2-low group in low-grade UC was significantly better than that of the PAR-2-high group (**A**). Whereas, no significance was observed in high-grade UC (**B**). The RFS of the matriptase-high group indicted a tendency toward a higher recurrence rate than that of the matriptase-low group in high-grade UC (**C**).

NMIBC. In addition, expression of matriptase indicated a tendency toward recurrence in high-grade UC, suggesting an important role of matriptase-induced PAR-2 activation in recurrence in NMIBC. However, since the number of specimens for this analysis was quite low, further examination using a larger number of specimens is required to confirm our results.

Disclosure

The authors report no conflicts of interest in this work.

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