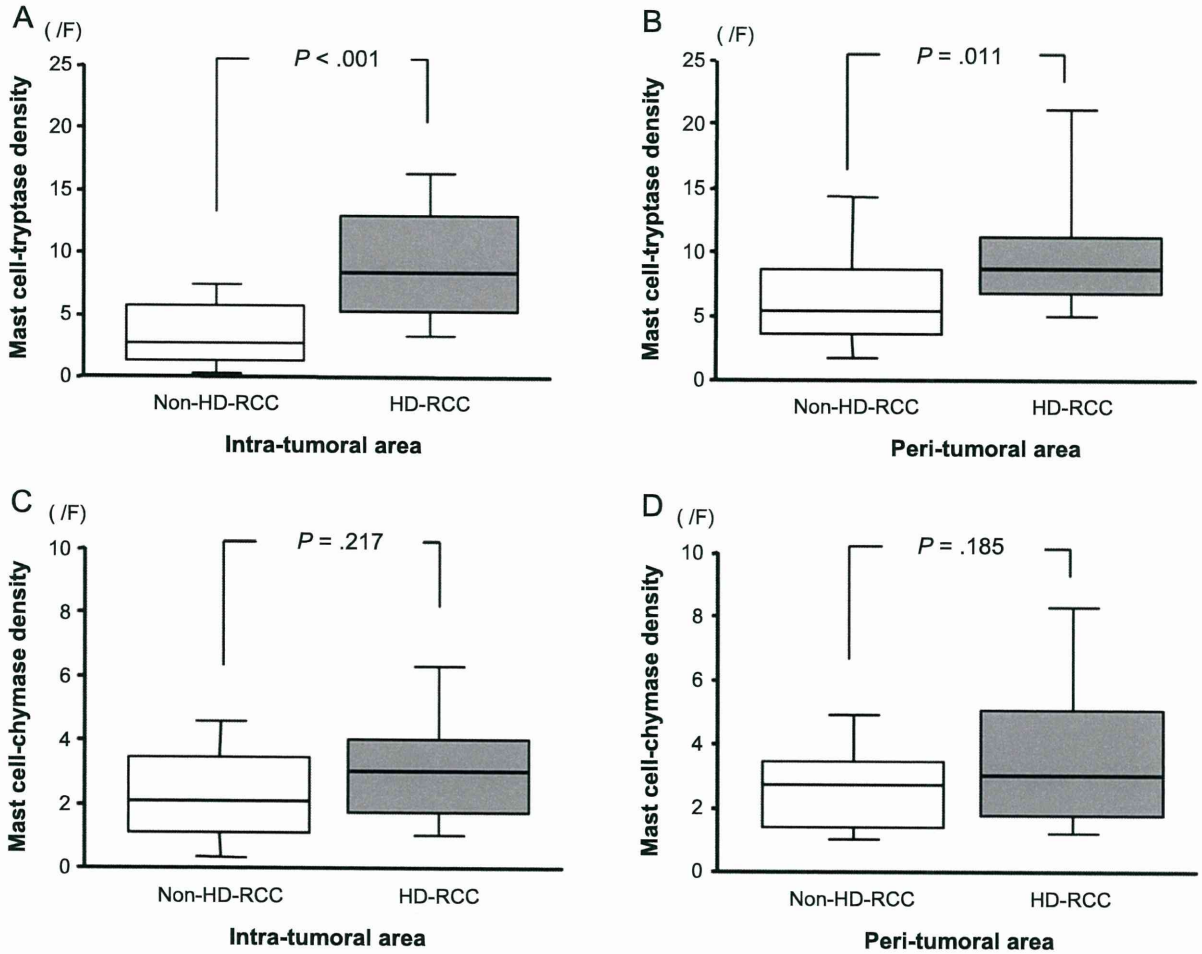

Fig. 2 Box-and-whisker plots of MC-t density (A and B) and MC-chymase density (C and D) in intratumoral and peritumoral areas of non–HD-RCC and HD-RCC. In these plots, lines within the boxes represent median values; the upper and lower lines of the boxes, the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes, the 90th and 10th percentiles, respectively.

3. Results

3.1. Densities of MC-t and MC-tc and expression levels of SCF and PAR-2

Figs. 1A to E shows representative examples of MC-t in normal kidney, non–HD RCC, and HD-RCC. The density of MC-t in the intratumoral area of HD-RCC (median, 8.3; IQR, 5.3-12.9 per HPF) was significantly higher than in the non–HD-RCC (2.7, 1.3-5.7 per HPF, $P < .001$, Fig. 2A). Likewise, the MC-t density in the peritumoral area was significantly higher in the HD-RCC (8.7, 6.8-11.2 per HPF) than in the non–HD-RCC (5.3, 3.5-8.6 per HPF, $P = .011$, Fig. 2B). In addition, MC-t density in each area was significantly higher than in the normal kidney (1.3, 0.4-2.8).

Figs. 1F, G, H, I, and J show representative examples of MC-tc in the normal kidney, intratumoral area of non–HD-RCC, peritumoral area of non–HD-RCC, intratumoral area of HD-RCC, and peritumoral area of HD-RCC, respectively. In contrast to MC-t density, there were no significant differences in MC-tc density between non–HD-RCC and HD-RCC in intratumoral ($P = .217$, Fig. 2C) and peritumoral areas ($P = .185$, Fig. 2D). On the other hand, there was no specific or characteristic distribution pattern for MC-tc based on the area of the tumor or

Fig. 1 A to C, Representative examples of MC-t in non–HD-RCC. MC-t in the normal kidney (A), intratumoral area (B), and peritumoral area (C). D to E Representative examples of MC-t in HD-RCC. MC-t in the intratumoral (D) and peritumoral areas (E). MC-t density in the intratumoral area of HD-RCC (D) is higher than that of non–HD-RCC (B). F to J, Representative examples of MC-tc. Normal kidney (F), intratumoral area of non–HD-RCC (G), peritumoral area of non–HD-RCC (H), intratumoral area of HD-RCC (I), and peritumoral area of HD-RCC (J). K to M, Representative examples of PAR-2 expression. Normal kidney tissue (K), tumor area of non–HD-RCC (L), and tumor area of HD-RCC (M). N to P, Representative examples of SCF. Normal kidney (N), non–HD-RCC (O), and HD-RCC (P) (original magnification ×200).

Table 2 Relationship between MC densities and pathologic features in HD-RCC

	No. of patients	MC-t density (IQR)			
		Intratumoral	*P*	Peritumoral	*P*
pT stage					
T1a	25	9.1(5.1-12.8)	] 0.757	8.0(6.6-10.8)	] 0.575
T1b	7	10.3(5.7-16.0)	] 0.472	9.7(7.0-20.8)	] 0.750
T2	3	7.5(4.9- 7.9)		10.7(8.2-11.1)	
Grade					
G1	15	8.3(3.5-12.4)	] 0.378	9.7(7.6-10.7)	] 0.587
G2	13	9.7(6.7-16.3)	] 0.771	9.0(6.2-17.9)	] 0.712
G3/4	7	8.0(5.9-12.3)		7.3(6.4-10.6)	
ACDK					
Without	10	11.0(5.2-16.3)	] 0.228	8.9(7.6-10.7)	] 0.812
With	25	7.7(5.1-11.7)		8.3(6.6-11.3)	

HD-RCC: renal cell carcinoma with hemodialysis, ACDK: acquired cystic kidney disease

histopathologic type. Finally, the MC-t densities in both intratumoral and peritumoral areas were significantly higher than MC-tc densities in the corresponding areas in both non–HD-RCC and HD-RCC (Fig. 2).

Figs. 1K, L, and M show representative examples of PAR-2 expression in normal kidney tissue, non–HD-RCC, and HD-RCC, respectively. PAR-2 was detected in parts of the normal tubular cells, and some tubules showed strong expression. However, PAR-2 was only weakly expressed in almost all tubules, and no strong expression was detected in normal cells. On the other hand, in cancer cells, moderate to strong expression was often found. Finally, in non–HD-RCC, 13 (33.3%) of 39 specimens were judged positive for PAR-2 expression. On the other hand, the proportion of PAR-2–positive cancer cells was 68.6% in HD-RCC. The proportion of PAR-2–stained cells was significantly higher in HD-RCC than in non–HD-RCC ($P = .003$).

Figs. 1N, O, and P provide representative examples of SCF in normal kidney, non–HD-RCC, and HD-RCC, respectively. SCF was mainly detected in the cell cytoplasm and part of the cell membrane. The proportion of SCF-positive cells was significantly higher in HD-RCC cells (62.9%) than non–HD-RCC (33.3%, $P = .035$). However, moderate or strong expression was relatively rare in normal renal tubular cells.

3.2. Clinical and pathologic significance of MC-t and PAR-2 expression

Table 2 summarizes the relationships between pathologic features and MC-t density in intratumoral and peritumoral areas in HD-RCC. There was no significant relationship among these factors and pT stage or grade. In addition, MC-t

Table 3 Relationship between PAR-2 and SCF and pathologic features in HD-RCC

	PAR-2 expression			SCF expression		
	Negative	Positive	*P*	Negative	Positive	*P*
pT stage			.983			.937
T1a	8 (72.7)	17 (70.8)		9 (69.2)	16 (72.7)	
T1b	2 (18.2)	5 (20.8)		3 (23.2)	4 (18.2)	
T2	1 (9.1)	2 (8.3)		1 (9.2)	2 (9.1)	
Grade			.203			.905
G1	7 (63.6)	8 (33.3)		5 (38.5)	10 (45.5)	
G2	2 (18.2)	11 (45.8)		5 (38.5)	8 (36.4)	
G3/4	2 (18.2)	5 (20.8)		3 (23.1)	4 (18.2)	
ACDK			.447			.055
Without	2 (18.2)	8 (33.3)		1 (7.7)	9 (40.9)	
With	9 (81.7)	16 (66.7)		12 (92.7)	13 (59.1)	

Table 4 PI in patients with HD

	PI (%)	*P*
Intratumoral MC-t		
Low	5.5 (4.6-9.3)	.039
High	10.4 (6.8-15.7)	
Peritumoral MC-t		
Low	9.3 (5.7-10.4)	.476
High	9.5 (5.3-15.3)	
PAR-2 expression		
Negative	5.5 (4.4-7.3)	.008
Positive	11.2 (6.9-16.0)	
SCF expression		
Negative	9.3 (5.4-11.9)	.573
Positive	8.9 (5.0-15.5)	

Data were shown as median (IQR).

density in intratumoral and peritumoral areas did not correlate with sex (P = .273 or .841) or age (P = .463 or .689). The duration of HD (median, 141; IQR, 74-213 months) did not correlate with MC-t in intratumoral (r = 0.04, P = .815) and peritumoral areas (r = 0.23, P = .201). The density of MC-t did not correlate with acquired cystic kidney disease (ACDK) (Table 2) or with pathologic type. Furthermore, the expression of both PAR-2 and SCF in HD-RCC cells did not correlate with various pathologic parameters (Table 3). On the other hand, the presence of ACDK tended to correlate with SCF expression, albeit insignificantly (P = .055, Table 3).

We also investigated the pathologic role of the above factors and proliferation index (PI) in HD-RCC (Table 4). PI correlated with MC-t density in the intratumoral area (P = .039) and PAR-2 expression (P = .008) but not with MC-t in the peritumoral area or SCF expression (Table 4). In addition, for the intratumoral area, PI was significantly higher in specimens showing PAR-2–positive and high–MC-t density (median, 12.9; IQR, 9.6-18.0 per HPF) than in those with PAR-2–positive and low–MC-t density or PAR-2–negative and high–MC-t density (6.8, 4.5-9.8, P = .011) and those with PAR-2 negative and low MC-t (5.0, 3.8-5.4, P = .037). To clarify in more detail the activities of MC-t in intratumoral area and PAR-2 expression, we investigated the relationship among these parameters and apoptosis. With regard to MC-t in intratumoral area, the proportion of TUNEL-positive cells in specimens with high–MC-t density (1.4, 1.1-2.4) was significantly (P = .014) lower than in those with low–MC-t density (2.3, 1.9-3.1). In addition, a similar trend was found in cleaved caspase-3–positive cancer cells (P = .181). On the other hand, there was no significant relationship between PAR-2 expression and TUNEL-positive (P = .776) or cleaved caspase-3–positive cells (P = .790).

Recurrence occurred in 4 patients with HD-RCC and 1 patient with non–HD-RCC. The disease-free survival was worse in HD-RCC (log-rank P = .034). In addition, among the 4 patients with HD-RCC who developed recurrence, the MC-t density in intratumoral area was high in 3 patients.

3.3. Relationship among MC-t density and expression of PAR and SCF in HD-RCC

Based on the above results, we further investigated the relationships among MC-t density in intratumoral area and the expression of SCF and PAR-2 in cancer cells in HD-RCC. MC-t density in SCF-positive specimens (median, 5.0; IQR, 10.0-15.0 per HPF) was significantly higher (P = .039) than in SCF-negative ones (7, 3.6-8.7 per HPF). In addition to SCF, MC-t density correlated positively with the density of macrophages in intratumoral area (r = 0.462, P = .005). On the other hand, SCF expression also correlated with PAR-2 expression (P = .356). Furthermore, the IRS of PAR-2 correlated with MC-t density (r = 0.378, P = .025).

4. Discussion

One of the unique results of our study is that MC-t density in HD-RCC was significantly higher than in non–HD-RCC, but no such difference was found in MC-tc. In addition, in HD-RCC, the MC-t densities in both intratumoral and peritumoral areas were significantly higher than MC-tc densities in similar areas. Based on these results, we speculate the dominance of MC-t relative to MC-tc and that MC-t plays a more important role in HD-RCC. Our findings add support to those of previous studies that showed a significantly higher MC-t density than that of MC-tc in non–small cell lung [13] and gastric cancers [30]. In this regard, a previous study using in situ staining reported the presence of tryptase and only low or undetectable amounts of chymase in human renal MCs derived from human renal tumors tissues [31]. These studies add support to the notion that MC-t may have a more significant contribution to the malignant potential compared with MC-tc under the pathologic conditions in RCC. In fact, our results showed that MC-t was associated with poor outcome in HD-RCC.

MCs have 2 paradoxical actions (procancer and anticancer) within the tumor microenvironment [5,6]. In the present study, the intratumoral density of MC-t correlated with cancer cell proliferation in HD-RCC. The involvement of tryptase secreted from MCs in cell proliferation has been reported also in various cancers such as colon and pancreas cancers [7,8]. On the other hand, our results showed no relation between MC-t and MC-tc and any of the pathologic parameters examined in patients with HD-RCC, in agreement with the results of previous reports on patients with RCC [14,15]. Although further studies are needed to examine the clinical significance of MCs in these patients, it should be noted that all patients of this study underwent ultrasonography and/or computed tomography of the kidney at 1 to 3 times every year. Thus, 25 (71.4%) of 35 patients with HD-RCC were diagnosed with pT1a. Tuna et al [14] analyzed the number of MC in 71 RCC patients and reported a pT1a rate of 23.9%. In addition, Mohseni et al [15] also

conducted a similar study in 40 patients, including those with 4 (10%) pT1a tumors. Thus, in our study population, tumors were detected in early stage, and there is a possibility that MC-t density was not associated with the pathologic features because of such high frequency of low-stage cancer. Interestingly, in tissues of 3 patients with HD-RCC who developed local invasion and/or metastasis, the MC-t density in the intratumoral area was markedly high (17.2, 20.0-21.0 per HPF). In addition, our study population was relatively small because it was performed in a single hospital.

Contrary to HD-RCC, a significant relation between MC-t and cancer cell proliferation was not found in non–HD-RCC. Our result also showed a significantly higher PI in HD-RCC than in non–HD-RCC (data not shown). This result suggests that the pathologic role of MC-t in HD-RCC is different from that in non–HD-RCC. To further clarify the role of MC-t in HD-RCC, we investigated PAR-2 expression in HD-RCC tissues because the biologic activities of PAR-2 are mediated by tryptase from MCs, and PAR-2 is known to be involved in the initiation of cell proliferation in various cancers [7,25,26,32]. Although PAR gene expression was previously reported in kidney cancer cell line (A-498) by reverse transcriptase–polymerase chain reaction [33], there is no information on PAR-2 protein expression in human RCC tissues. In our study, PAR-2–positive cells were rarely found in normal renal tubules, adding support to the results of a previous report [27]. On the other hand, PAR-2 expression was detected in approximately one third of the non–HD-RCC tissues. Thus, we speculate that PAR-2 expression might be up-regulated by the carcinogenic process of RCC. In addition, our results also showed the expression of PAR-2 in approximately two thirds of HD-RCC tissues and that it was significantly higher than in non–HD-RCC. Although the in vivo physiologic and pathologic roles of PAR-2 remain poorly understood, previous studies reported that PAR-2 expression correlated with serum creatinine levels and tubulointerstitial fibrosis in various types of nephropathies [20,27]. Furthermore, PAR-2 is mainly expressed within the proximal tubular cells in specimens of nephropathy [27]. Considered together, the above results suggest that PAR-2 expression is up-regulated by progression of nephropathy. On the other hand, 1 study reported that MCs-released tryptase stimulated PAR-2 expression in colon cancer cells in a paracrine manner [7]. Our results also showed that MC-t density correlated significantly with PAR-2 expression in HD-RCC. Thus, it is possible that trypsin released from MC-t stimulates PAR-2 expression in HD-RCC in a paracrine manner similar to colon cancer. On the other hand, our results showed that MC-t density, but not PAR-2 expression, correlated negatively with apoptosis in HD-RCC. Although we did not explore the main reason for such difference, it seems that the apoptotic function of MC-t in HD-RCC is modulated by PAR-2–independent pathway.

Another key factor investigated in the present study is SCF. This factor is essential in almost all biologic functions of MCs including cell differentiation, proliferation, survival, recruitment, and secretion under various pathologic conditions including malignancies [18,22]. Our results showed a higher SCF expression in HD-RCC tissues compared with non–HD-RCC. This result may explain the finding of significantly higher MC-t density in HD-RCC relative to non–HD-RCC. What is the mechanism underlying SCF up-regulation in HD-RCC compared with non–HD-RCC? Although the exact mechanism remains obscure, we speculate that 2 different mechanisms may affect the expression of SCF in HD-RCC tumors. First, the progression of nephropathy and worsening of renal dysfunction correlates with SCF function [19,27]. For example, SCF expression was found to correlate with serum creatinine level and renal tubular fibrosis. Furthermore, serum SCF levels were reported to be 5-fold higher in patients with ESRD than in healthy controls [23]. Second, cancer cells and/or stromal cells may enhance SCF expression by changing the tumor microenvironment. This notion is based on 2 facts: cultured RCC cells (A-498, BFTC-909, CAKI-1, CAKI-2, and CAL-54 cell lines) did not secrete SCF when incubated in standard media [34] and hypoxia in the local tumor microenvironment and hypoxia-inducible factor can directly promote SCF expression in breast cancer cells [35].

The biologic functions and pathologic roles of MC vary with changes in the microenvironment by inflammatory-associated factors including cytokines and growth factors [3]. Likewise, the kidney of patients with HD is also exposed to these inflammatory-associated factors. Our study showed a significantly higher density of macrophages in HD-RCC relative to that in non–HD-RCC (data not shown). Thus, the pathogenesis and malignant aggressiveness of RCC in patients on HD seem to be regulated by complex mechanisms. The pathologic significance and function of MCs in HD-RCC tumors is also considered to be regulated by a panel of different and complex mechanisms. Further studies, including cell culture experiments, are needed to determine the clinical significance and define the regulatory mechanism(s) that control MCs in patients with RCC with HD.

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Clinical Significance and Predictive Value of Prostaglandin E2 Receptors (EPR) 1 – 4 in Patients with Renal Cell Carcinoma

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Abstract. *Background: The clinical significance of prostaglandin E2 receptor (EPR) expression in renal cell carcinoma (RCC) tissues remains unclear. Patients and Methods: Four subtypes of EPRs were examined in 112 human RCC tissues by immunohistochemical and Western blot analysis. The relationships between EPR immunoreactivity score (IS) and various pathological features and survival were then analyzed. Results: The IS of EP4R was significantly higher ($p<0.001$) in cancer cells (mean=2.7 and SD=2.1) than in normal kidney tissues (1.8 and 1.2). EP4R expression correlated with pT stage, metastasis, and grade. EP2R expression was also associated with metastasis. Expressions of both EP2R and EP4R were found to be significant predictors for cause-specific survival on Kaplan-Meier survival analysis ($p=0.006$ and 0.023, respectively). Conclusion: EP2R and EP4R may play important roles in malignant behavior. EP4R in particular was closely associated with pathological features, implicating this receptor as a potential therapeutic target in patients with RCC.*

Cyclooxygenase (COX)-2 plays crucial roles in carcinogenesis, tumor growth, and progression of various malignancies (1-4). In addition, COX-2 expression in cancer cells is up-regulated compared to adjacent normal cells in several human tissues (5-8). COX-2 has therefore been proposed as a useful therapeutic target in malignancies. Indeed, COX-2 inhibitors may reduce the risk of carcinogenesis in several types of cancer including colon (9), lung (10), and prostate cancer (11), and are also further expected to have anti-tumorigenic effects. However, COX-2 inhibitors may increase the risk of cardiovascular disease including myocardial infarction (12, 13). Whilst such opinions are controversial, many oncologists are hesitant to use COX-2 inhibitors clinically because of the risk of limited success and severe side-effects. Thus, more effective and safer strategies are still needed to inhibit tumorigenesis.

COX-2 is a key enzyme in the conversion of arachidonic acid to prostaglandins (PGs), of which PGE 2 is the most well known due to its potent biological activity. Like COX-2, PGE 2 has also received attention in recent years because it is overexpressed in various cancer tissues and is associated with tumor growth and progression (14-16). Previous pharmacological and animal studies reported that the major antitumor action of COX-2 inhibitors is mediated through inhibition of PGE 2 (17, 18). Thus, further understanding of the pathological function of PGE 2 would also contribute to future cancer treatment strategies.

The biological activities of PGE 2 are mediated through their respective receptors, which are called E prostanoid receptors (EPRs) and are divided into four subtypes: EP1R, EP2R, EP3R, and EP4R (19). Several studies have associated tumor development and progression with EPR expression levels in various malignancies (20-24), with the EPR expression pattern varying with cancer type. For example, EP1R expression has been linked to carcinogenesis and tumor development in colon (17), breast (25), and prostate cancer (26), while PGE 2 was implicated in modulating cancer cell function *via* EP2R and EP4R activities in endometrial adenocarcinoma (23) and cervical adenocarcinoma (24). Another study also demonstrated EP3R expression as a critical factor in PGE 2-mediated tumor development in lung adenocarcinoma (21). EP4R is also thought to contribute to tumor growth, progression

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and/or carcinogenesis in colon cancer (27), gallbladder cancer (22), and in transitional cell carcinoma of the upper urinary tract (28). To our knowledge, EPR expression has not been reported in human renal cell carcinoma (RCC), and any relationship between clinicopathological features or prognosis and expression of EPRs remains unknown.

In recent years, various molecular targeting therapies including antiangiogenic agents have been used for patients with RCC, especially those with advanced tumors. However, although such agents often show high antitumoral effects compared to conventional therapies, many oncologists and urologists are still seeking new antitumoral strategies because resistance to such therapies typically develops within 12 months (29, 30). The present study was designed to clarify the significance of EPR expression in human RCC tissue. We also investigated the relationship between EPR expression and various clinicopathological features and survival in patients with RCC. The findings of this study have important implications for developing new preventative and treatment strategies for RCC patients.

Patients and Methods

Patients. The subjects of the present study were 112 consecutive patients who underwent radical nephrectomy for RCC at Nagasaki University Hospital from 1992 to 2005. Patients who received neoadjuvant therapy, such as immunotherapy, or those with sarcomatous RCC were excluded from the study. In addition, some patients refused preservation of renal tissues in any other form except for formalin-fixation and paraffin embedding, and were thus excluded from the study. All patients underwent preoperative ultrasonography, computed tomography of the abdomen, bone scanning, and lung radiography to identify metastases. Magnetic resonance imaging (MRI) of the bone and abdomen and computed tomography of the lung and brain were performed as necessary. Pathological staging was assessed by the 2002 tumor node metastasis (TNM) classification, with the grade determined using the criteria of Fuhrman *et al.* (31). Several pathologists performed the pathological examinations, with the final diagnosis judged by the chief pathologist (T.H.). We also examined EPR expression in 30 samples of normal kidney obtained from adjacent regions at least 2 cm from the tumor margins. In a preliminary study, we confirmed similar expression levels for EPRs in 30 pathological specimens and 20 normal kidney tissues free of hydronephrosis (obtained surgically from patients with ureteric tumors). The Human Ethics Review Committee of Nagasaki University Hospital approved the study protocol.

The study subjects were 79 males and 33 females with a mean (SD) age at surgery of 60.1 (12.3) years. Among these 112 patients, 87 (77.6%) had low pT stage (pT1=69 and pT2=18 patients) and 25 (22.3%) had high pT stage (pT3=24 and pT4=1 patients). Six patients had lymph node metastases, 17 had distant metastases; and 5 patients had both lymph node metastases and distant metastases. With regard to the nuclear grade, there were 52 tumors in G1, 48 in G2, 11 in G3, and 1 in G4. As for prognostic implications, 17 patients (15.2%) died of RCC during the follow-up period (mean=53.3, SD=36.8 months).

Immunohistochemistry. Five-μm-thick sections from formalin-fixed and paraffin-embedded specimens were deparaffinized in xylene and rehydrated. For all antibodies, antigen retrieval was performed at 95°C for 40 minutes in 0.0 1 M sodium citrate buffer (pH 6.0). All sections were then immersed in 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. All primary antibodies of EPRs were obtained from Cayman Chemical Corporation (Ann Arbor, MI, USA). Sections were incubated with primary antibodies at 4°C overnight. The sections were then treated with peroxidase using the labeled polymer method with Dako EnVision+TM Peroxidase (Dako Corp, Carpinteria, CA, USA) for 60 minutes. The peroxidase reaction was visualized using a liquid DAB substrate kit (Zymed Laboratories, San Francisco, CA, USA). A consecutive section from each sample processed without the primary antibody was used as a negative control. The normal renal tissue samples served as a positive control for the EPRs. In addition, control sections were also incubated with antisera in the presence of a 100-fold excess of human recombinant EPR protein (Cayman Chemical).

Evaluation. Three to five representative areas of each slide were evaluated, including at least 500 cancer cells and 200 proximal tubular cells according to a method described previously (32). In brief, the staining intensity was scored on a semiquantitative four-point scale as follows: 0, negative; 1, weak; 2, moderate; 3, strong. In addition, a semiquantitative estimate of the percentage of immunoreactive cells was determined using a scale of 0-4 (0, no staining; 1, 1-10% cells stained; 2, 11-50% cells stained; 3, 51-80% cells stained; 4, 81-100% cells stained). Finally, values for the quantity and staining intensity scores were then multiplied, giving results that ranged from 0 to 12 (immunoreactivity score=IS). These analyses were carried out using a Nikon E-400 microscope and digital images were captured using a digital camera (Nikon DU100, Japan) at ×200 magnification. In addition, a computer-aided image analysis system (Win ROOF, version 5.0, MITANI Corp, Japan) to calculate these variables. Slides were evaluated twice at different times by three investigators (K.O., Y.M., and S.W.) who were blinded to the pathological characteristics, and average levels were used for statistical analyses. Prostate cancer tissue with confirmed high expression of EPRs from a previous study (26) was used as positive control. A consecutive section from each sample processed without the primary antibody was used as a negative control.

Western blot analysis. Immunohistochemical staining of each EPR was confirmed by Western blot analysis of part of the same specimen. In this study, frozen tissues preserved at –80°C were used. They were firstly examined under a microscope to ensure that at least 90% of each sample comprised tumor or that they included normal glomerular and tubular cells. Tissues were thawed in ice-cold lysis buffer [150 mM NaCl, 100 mM Tris (pH 8.0), 1% Tween 20, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin, and 10 μg/ml leupeptin], sonicated for 3 min on ice, and then centrifuged at 10,000 × g for 10 min at 4°C to sediment the particulate material. Protein concentrations were measured using Bio-Rad (Hercules, CA, USA) reagent. Electrophoresis was carried out using 10% polyacrylamide gels with 4.5% stacking gel before transfer to nitrocellulose membranes for immunoblotting. The membranes were blocked in 5% skim milk in TBS and 0.1% Tween 20, and then incubated with the primary antibody (Cayman Chemical Corporation) overnight at 4°C. The membranes were treated with horseradish peroxidase-conjugated

Table I. *Relationships among immunoreactivity scores for EPRs and various pathological features.*

	EP1R	*P*-value	EP2R	*P*-value	EP3R	*P*-value	EP4R	*P*-value
pT stage								
T1	2.4 (2.0)		3.0 (1.9)		2.6 (1.9)		2.0 (1.8)	
T2	2.8 (1.7)	0.757	3.3 (1.5)	0.707	3.4 (1.7)	0.251	3.2 (2.0)	**0.036**
T3+4	2.8 (1.7)	0.999	2.5 (1.7)	0.857	2.8 (1.7)	0.635	4.4 (1.7)	0.117
Metastasis								
Absence	2.4 (1.9)		3.0 (1.8)		2.6 (1.9)		2.5 (2.0)	
Presence	3.3 (1.5)	0.073	4.2 (1.2)	**<0.001**	3.4 (1.5)	0.090	4.5 (1.3)	**<0.001**
Grade								
G1	2.2 (2.0)		2.8 (1.9)		2.4 (2.1)		1.8 (1.8)	
G2	2.8 (1.7)	0.260	3.3 (1.7)	0.350	2.9 (1.7)	0.400	3.2 (2.0)	**0.004**
G3+4	3.3 (1.8)	0.650	4.4 (1.0)*	0.136	3.4 (1.2)	0.743	4.7 (2.4)**	**0.028**

EPR, E prostanoid receptor. Data are the mean±(SD). *G1 *vs.* G3: p=0.014; **G1 *vs.* G3: p<0.001.

secondary antibody for 1 hour. Protein detection was performed with an enhanced chemiluminescence (ECL) kit according to the manufacturer's protocol. Levels of EPR protein expression were normalized to that of β-actin, which was used as a loading control.

Statistical analysis. All data are expressed as mean±SD. Student's *t*-test was used for the analysis of continuous variables and Scheffé's test was used for multiple comparisons of the data. The chi-square test was used for categorical comparison of the data. Survival analysis was evaluated by Kaplan-Meier analysis and the log-rank test. Variables that achieved statistical significance (p<0.050) by univariate analysis were subsequently entered into a multivariate analysis using COX proportional hazard survival analysis (described as odds ratio [OR] with 95% confidence intervals [95% CI], together with the p-values). For statistical evaluation, pT stage and grade was divided into two groups: low pT stage, pT1 and 2; high pT stage, pT3 and pT4; and low grade, G1 and 2; high grade, G3 and 4. In addition, to evaluate the predictive value of each EPR by statistical analyses, IS values were divided into two groups: negative (median or less than median IS) and positive (above median IS). All statistical tests were two-sided and significance was defined as p<0.050. Analyses were performed on a personal computer with the statistical package StatView for Windows (version 5.0) .

Results

Expression of EPRs. Representative examples of immunohistochemical staining of each EP receptor are shown in Figure 1. All EPRs were mainly detected in the cell membrane and cytoplasm. There was no clear difference in EP1R and EP3R expression (Figure 1A, B, E, F) between normal renal tubular tissue (A) and RCC cells (B); the mean (SD) IS in normal cells and cancer cells was 2.4 (1.2) and 2.6 (1.9), respectively (p=0.356) for EP1R and 2.6 (1.3) and 2.8 (1.9), respectively for EP3R (p=0.547). EP2R expression in normal cells (mean IS=2.9 and SD=1.4, Figure 1C) tended to be lower than that in cancer cells (3.2 and 1.8, Figure 1D), although this difference did not reach significance (p=0.174). For EP4R expression, the mean (SD) IS was 1.8 (1.2) in normal tubules and 2.7 (2.1) in cancer cells (Figure 1G, H); which was significantly higher (p<0.001). In addition to normal tubular cells and cancer cells, some infiltrating cells, fibroblast-like cells, and endothelial cell were also immunopositive for all EPRs. However, we noticed no common feature or unique distribution for any of the EPRs.

There was no significant difference with respect to the relationship between histological type and EPR expression (Figure 2). The mean IS for EP1R in chromophobe RCC [3.1 (1.4)] was higher than that in conventional RCC [2.5 (1.9)], but this difference was not significant (p=0.620). Similarly, the IS for EP2R, EP3R, and E4R in papillary RCC was higher than that in conventional RCC, although again the differences were not significant (p=0.185, 0.225, and 0.182, respectively). Western blot analysis confirmed the up-regulation of EP4R in RCC tissues as indicated by the IS values (Figure 3). Normal tissue no.2, which had the highest IS (=9), had the strongest band by Western blot. However, this was the only normal tissue with such high IS.

Correlation with pathological features. Table I details the relationships between various pathological features and expression of each EPR in cancer cells. The IS of EP1R and EP3R was not associated with any pathological feature, including pT stage, presence of metastasis, and grade. On the other hand, IS of EP2R expression was significantly associated with the positive status of metastasis (p=0.001). In addition, the IS in G3 tumors was significantly higher than that in G1 tumors (p=0.014), whereas such a difference was not found between G1 and G2 or G2 and G3/4 tumors. Significant associations were found between EP4R expression and both the presence of metastasis and high grade. In addition, this score in pT2 tumors was significantly higher (p=0.036) than that in pT1 tumors, but there was no significant difference between p2 and pT3+4 (p=0.117).

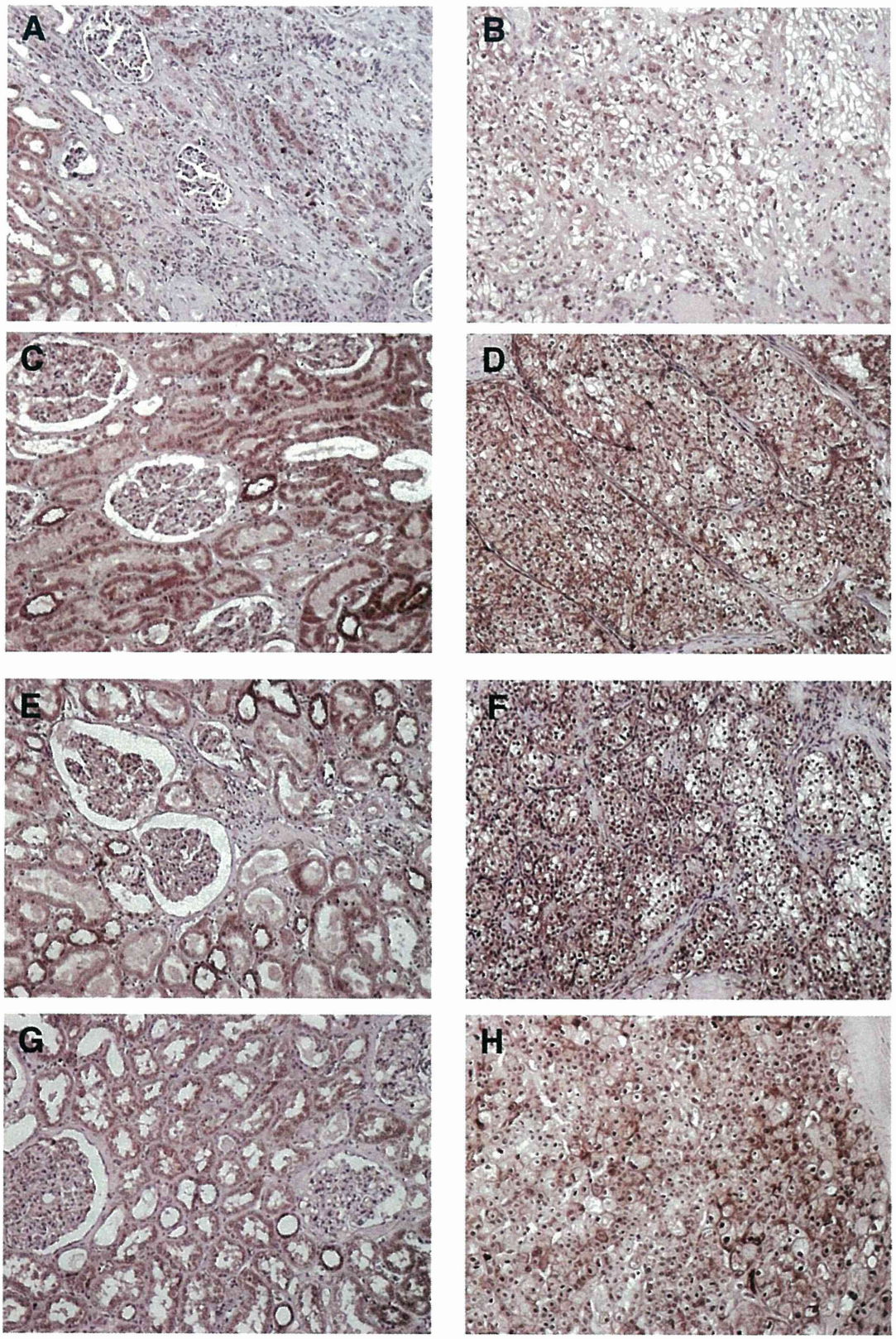

Figure 1. *The expression of each EP receptor was evaluated by immunohistochemistry. EP1R expression in normal renal tubular cells (A) and renal cancer cells (B); EP2R in normal cells (C) and cancer cells (D); EP3R in normal cells (E) and cancer cells (F); EP4R in normal cells (G) and cancer cells (H). EPR immunoreactivities were mainly detected in the cytoplasm. Magnification, ×200.*

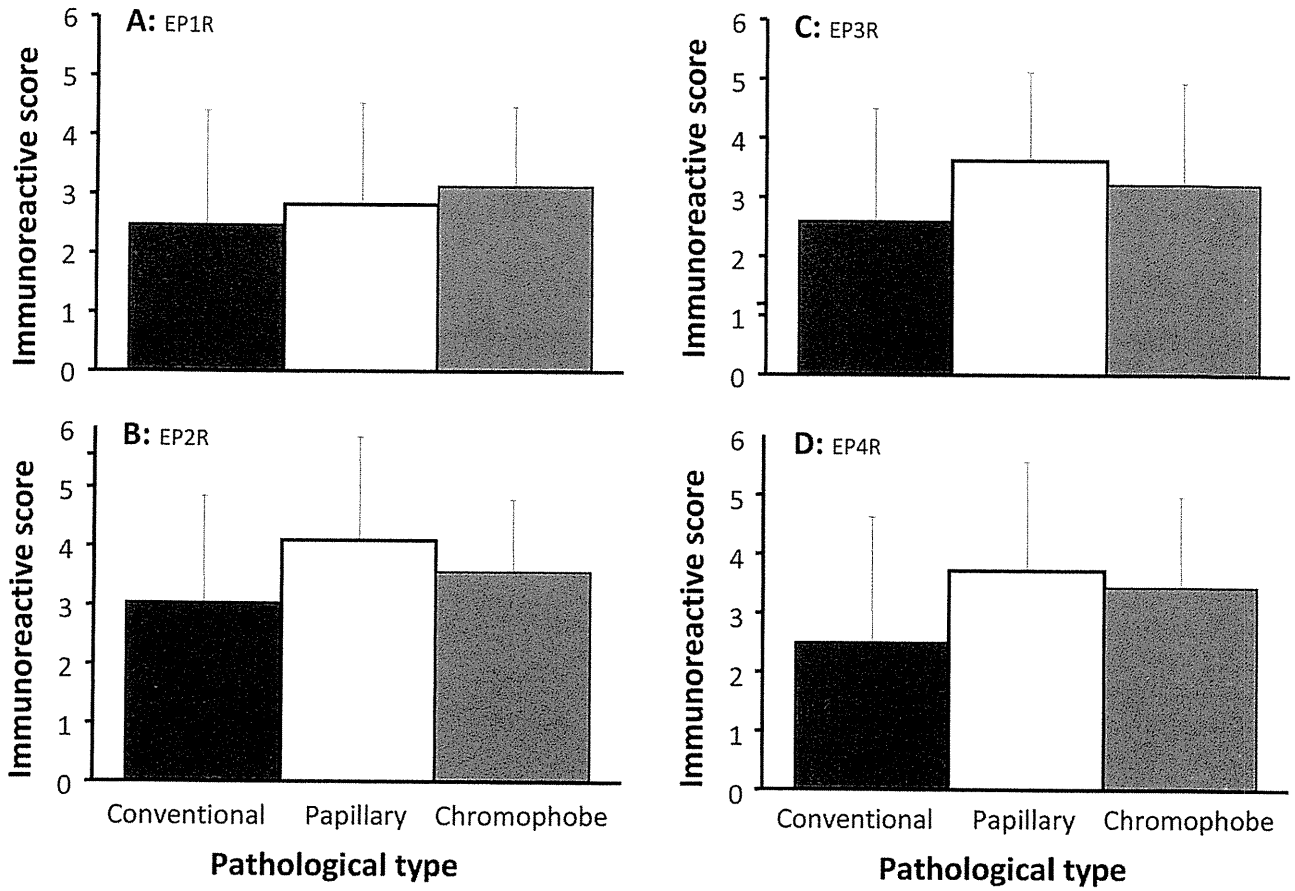

Figure 2. *Relationships among mean (SD) immunoreactivity scores of EPRs and pathological types of renal cell carcinoma. No significant differences were established. Data are mean±SD.*

Predictive values for survival. Based on the above results, we then analyzed the predictive values of EP2R and ER4R expression for cause-specific survival in patients with RCC. As shown in Figure 4A, patients with EP2R-positive cells had worse survival than patients with EP2R-negative cells (log-rank p=0.006). Similarly, EP4R expression was associated with poorer cause-specific survival (log-rank p=0.023). No such significant relationship for survival was evident for EP1R expression (log-rank p=0.541) and EP3R expression (log-rank p=0.179). In this study, 37 patients were treated with immunotherapy after surgery. However, this factor did not affect cause-specific survival (log-rank p=0.143). Likewise, age and sex were not recognized as significant predictors of survival by similar analyses. On the other hand, pT stage, presence of metastasis, and grade were all identified as strong and significant predictive factors (p<0.001). Multivariate analysis including the above pathological factors showed that neither EP2R nor EP4R expression was a significant predictor (hazard ratio=2.9, 95% confidence internal=0.8-10.5, p=0.112; HR=1.3, 95% CI=0.3-5.4, p=0.700, respectively). Only the presence of metastasis was an independent and significant predictor of cause-specific survival in patients with RCC (HR=11.8, 95%CI=3.2-44.2, p<0.001).

Discussion

This is the first study to investigate the clinical significance of *EPR* expression in human RCC tissues. In fact, no detailed localization or expression analysis of any EPR in normal kidney tissues including proximal renal tubules has been reported. Recently, Herman *et al.* (33) detected *EPR* mRNA expression in proximal renal tubule cells, reporting that *EP4R* expression was significantly lower compared to that of the other *EPRs* (33). Our current results for normal kidney tissues showed a similar trend. We also showed that only EP4R expression was significantly different when compared between normal cells in proximal tubules and cancer cells, and that the IS for EP4R was positively associated with tumor grade. Therefore, we speculate that EP4R expression is important for carcinogenesis and malignant potential in patients with RCC, which is consistent with previous reports in other types of

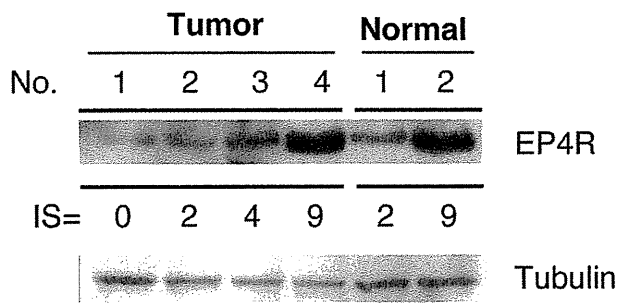

Figure 3. *Representative results of western blot analysis of EP4R expression in normal kidney and renal cell carcinoma tissues. The results were consistent with the determined immunoreactivity scores (IS).*

cancer (28, 34-36). However, it was surprising to find no significant difference in EP4R expression between pT2 and T3 and higher tumors, particularly given the previously reported correlation between enhanced expression of EP4R and cancer cell invasion in various types of cancer including ovarian and breast cancer (37, 38). In addition, EP4R signaling was reported to play a central role in matrix metalloproteinase-2-mediated malignant invasion (39), a process also known to be at play in human RCC tissues (40). Therefore, we had expected that EP4R expression in RCC tissues with invasion would be significantly higher than in those without invasion. The difference in the results might have been due to differences in the study designs such as number of patients, study population, and method of evaluation. Further research is required to clarify the pathological significance of EP4R in invasion of RCC cells.

Among the EPRs, EP2R is the most representative and well-known stimulator of tumor development and progression in various types of cancer based on previous reports (41-43). However, our results showed no significant difference in EP2R expression between normal and RCC tissues, and no association with pT stage. On the other hand, EP2R expression in metastasized tumor was significantly higher than in tumor without metastasis, and the expression in G3 tumor was significantly higher than in G1 tumor. Our results cannot explain this apparent contradiction. However, EP2R was reported to be associated with metastasis in lung cancer (44). Interestingly, one study showed that up-regulated EP2R signaling enhanced lymph node metastasis in breast cancer cells (38), while another associated EP2R overexpression with depth of invasion, but not with metastasis, in esophageal carcinoma (45). Thus, the pathological role of EP2R seems to be cancer-type dependent, and the tissue-specific effects probably reflect how EP2R expression and function are regulated. We maintain that EP2R plays a significant role in malignant potential, especially for metastasis in some tumor types.

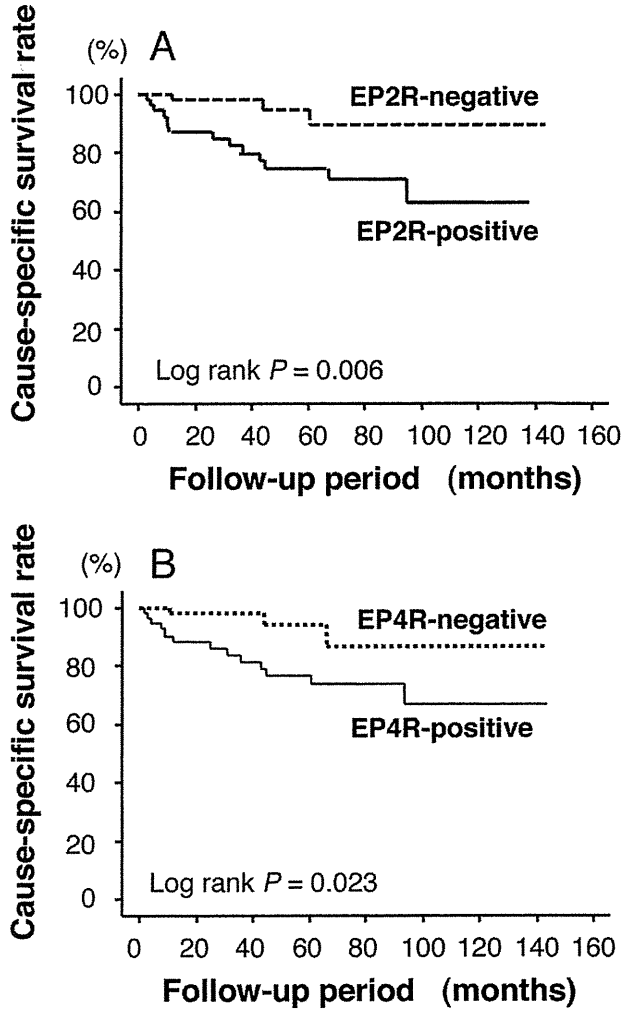

Figure 4. *Kaplan-Meier survival curves for EP2R (A) and EP4R for cause-specific survival.*

With regard to the relationship between EPR expression and cause-specific survival, the results identified EP2R and EP4R expressions but not EP1R or EP3R as being significant predictors of survival in patients with RCC. Our findings are in accord with previous reports in other cancer types (44, 45). Based on our demonstrated association of EP4R expression with pT stage and presence of metastasis, the survival analysis result for EP4R expression was expected and conceivable. However, the basis for EP2R expression as a useful predictor of survival was less clear, but we speculate that correlation with metastasis is the cause of such a phenomenon. In fact the presence of metastasis is closely associated with cause-specific survival, and it is only considered to be an independent predictor by multivariate analysis. On the other hand, the expressions of EP2R and EP4R were not recognized as significant and independent predictors by a multivariate analysis model that included

presence of metastasis. Thus, the presence of metastasis is a strong predictor for cause-specific survival in our study population of RCC, and this may have affected our results regarding survival.

Expressions of EP1R and EP3R in cancer cells were not significantly different from those in normal cells, and were not associated with clinicopathological features. In addition, they were not useful predictors of cause-specific survival. These results suggest that EP1R and EP3R are not important players in malignant behavior or prognosis in patients with RCC.

Interestingly, the EPR expression in this study did not correlate with the RCC histological type. There is general agreement that carcinogenic processes and malignant aggressiveness are affected by specific factors in some histological types such as the von-Hippel Lindau (*VHL*) gene in conventional RCC and *c-Met* in papillary RCC, and a previous study implicated EP1R in activating c-Met phosphorylation with enhanced cell invasion in human hepatocellular carcinoma (46). However, these factors do not seem to affect EPR expression in human cancer tissues. Finally, although our results did not address the mechanisms involved in EPR regulation, their expressions in RCC cells were unlikely to depend on the *VHL* gene or c-Met system.

Various trials and novel strategies are currently underway regarding the treatment of RCC, especially for advanced tumors, and the effective periods of almost all antiangiogenic drugs are typically one year (29, 30). Based on our results, EP2R and EP4R could both provide potential new therapeutic targets for RCC. However, we expect that EP4R would be a more effective and safer target in patients with RCC based on the lower EP4R expression in normal kidney tissues compared to RCC cells and the potential role of EP4R, but not EPR2, in malignant aggressiveness. Recent efforts to target the EP4R, such as by its selective inhibition, have demonstrated reduced tumor growth and metastasis (47, 48). Based on these previous reports and the present study, we therefore suggest selective inhibition of EP4R as the most promising potential therapeutic target in patients with RCC, but stress that inhibition of EP2R might also be useful in some RCC patients, such as in those with metastasis.

In summary, our investigation of the clinical and pathological significance of EPR expressions in human RCC tissue indicated that EP4R expression was associated with tumor growth and metastasis. Likewise, EP2R expression correlated with metastasis. Expression of EP2R and EP4R were also useful predictors of cause-specific survival. Based on these results, EP2R and EP4R might play important roles in tumor development and progression in patients with RCC. Furthermore, ER2R and EP4R could be used as potential therapeutic targets in RCC.

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Conflict of Interest Statement

We declare that we have no conflict of interest.

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Pathologic Significance and Prognostic Value of Phosphorylated Cortactin Expression in Patients With Sarcomatoid Renal Cell Carcinoma

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OBJECTIVES To clarify the clinical and prognostic significance of cortactin and phosphorylated cortactin in patients with sarcomatoid renal cell carcinoma (SRCC).

METHODS We retrospectively reviewed the data from 31 patients with SRCC and 33 with conventional renal cell carcinoma matched for clinicopathologic features. The immunoreactive score for cortactin, pY421 cortactin, and pY466 cortactin were measured using immunohistochemistry. The relationships between each immunoreactive score and the clinicopathologic features and survival were investigated.

RESULTS The immunoreactive score of p421 cortactin, but not that of cortactin and pY466 cortactin, was significantly greater in SRCC than in conventional renal cell carcinoma ($P < .001$). The expression of pY421 cortactin in SRCC correlated with the pT stage and metastasis ($P < .001$). The expression of pY466 cortactin showed a similar trend with pT stage ($P = .043$) but not with metastasis. Although both of pY421 cortactin and pY466 cortactin were identified as useful predictors for survival in univariate analyses, only pY421 cortactin expression was considered an independent predictor in patients with SRCC (odds ratio 4.53, 95% confidence interval 1.07-19.12, $P = .040$) in the multivariate analysis model, including pT stage and metastasis.

CONCLUSIONS Our results have demonstrated that phosphorylation of cortactin is a key process in malignant aggressiveness, and its expression is a useful predictor of cause-specific survival and could be a useful potential therapeutic target in patients with SRCC. UROLOGY 78: 476.e9–476.e15, 2011.

Sarcomatoid renal cell carcinoma (SRCC) originates from dedifferentiated renal cell carcinoma (RCC). Although this tumor is relatively rare, understanding its pathologic features and predictors of survival is important, because SRCC is characterized by local aggressiveness and high metastatic potential and is associated with an extremely poor prognosis. The disease-specific 5- and 10-year survival rate has been reported to be only 22% and 13% after primary treatment, respectively.[1] The mechanisms involved in the malignant aggressiveness of SRCC have been investigated in morphologic, chromosomal, and protein analysis studies,[2] although we do not yet have sufficient data to discuss strategies for follow-up and treatment.

Cortactin, which is encoded by the excess microsporocytes (EMS) 1 gene, is a multidomain protein originally identified as a major substrate of Src.[3] Cortactin regulates the dynamic actin networks and organization and the functioning of cell adhesion structures in many cellular processes, encompassing cell migration and adhesion in various physiologic responses and pathologic conditions.[4,5] Such actin-based cellular processes are also important in cancer cell invasion and metastasis. Cortactin is known to promote cancer cell invasion, and its overexpression is associated with tumor development, metastasis, and prognosis in a variety of cancers.[6-10] However, other investigators found that tumor growth and size did not correlate with cortactin expression in breast and hepatocellular carcinoma.[11,12] Thus, opinions are still conflicting regarding the biologic roles and pathologic significance of cortactin.

Various stimuli, including growth factors, cell adhesion, and hyperosmotic stress can induce tyrosine phos-

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phorylation of cortactin.[5] Protein phosphorylation serves as a major switch for many cellular signaling events, and the same applies to cortactin. Previous studies reported that increased levels of phosphorylation enhance cortactin-mediated functions, such as cell migration, invasion, and metastasis.[11,13,14] In contrast, the opposite finding (ie, hyperphosphorylated cortactin inhibits cell motility) were reported in several cancer cells, including gastric and breast cancer.[15] Thus, the pathologic roles of phosphorylated cortactin are not fully understood. In this regard, 1 study discussed the pathologic roles of phosphorylated cortactin in vivo[11] and another of RCC highlighted the clinical significance of cortactin in patients with RCC, including SRCC,[16] but did not mention the role of phosphorylated cortactin. Thus, research on the clinical significance and prognostic role of phosphorylated cortactin is important to define its true pathologic role in various malignancies, including SRCC.

Previous reports have shown that phosphorylation is regulated by several factors, have mapped various phosphorylation sites, and have indicated that Y421 and Y466 (corresponding to Y421 and Y470 in human cortactin) are the most representative and most studied sites, because they are targets of a variety of kinases and growth factors.[13,17-19] In human SRCC, the pathologic significance of cortactin expression and phosphorylated cortactin is not yet fully understood, in part, because a useful cell line has not yet been established. The main purpose of the present study was to determine the expression of cortactin and phosphorylated cortactin in human conventional renal cell carcinoma (CRCC) and SRCC tissues using site-specific antibodies against cortactin phosphotyrosine 421 and 466. In addition, the relationships between their expression levels and pT stage, metastasis, and survival were also analyzed and assessed. The results indicated enhanced phosphorylation of cortactin at Y421 residue in SRCC and that its expression correlated with tumor progression and the prognosis of patients with SRCC. These results suggest that pY421 cortactin expression is a useful prognostic marker for survival and a potential therapeutic target in patients with SRCC.

MATERIAL AND METHODS

The medical records of 31 patients diagnosed with SRCC from 1991 to 2009 were retrospectively reviewed. In the present study, we included SRCC derived from conventional RCC (CRCC) only. To match the clinicopathologic features, 33 patients diagnosed with grade 4 CRCC during the same period were selected and reviewed. We excluded patients with papillary RCC, chromophobe RCC, or unclassified tumors, patients receiving hemodialysis, and those with other cancers. Staging was assessed using the 2002 TNM classification. The nuclear grade was determined using the criteria of Fuhrman et al.[20] To assess tumor metastasis, all patients underwent ultrasonography, computed tomography of the abdomen, bone scanning, and pulmonary radiographic photography. Magnetic resonance imaging of the bone and abdomen and computed tomography of the lung and brain were performed as necessary. The tumors were divided into 2 groups according to the tumor stage: low-stage (Stage T1 or T2) and high-stage (Stage T3 or T4) tumors. Two pathologists performed all the pathologic examinations, and the final diagnosis was approved by the chief pathologist (T.H.). All patients with SRCC received immunotherapy, including interferon and/or interleukin-2 after surgery. The present study included no patients with SRCC treated by molecular-targeted therapy. Of the patients with CRCC, 29 (87.9%) received the same immunotherapy regimen, 2 were treated with sorafenib, and 2 received no additional treatment. The study design was in accordance with the guidelines of the human ethics review committee of Nagasaki University Hospital.

Tissue sections (5 μm thick) from formalin-fixed and paraffin-embedded specimens were deparaffinized in xylene and rehydrated. Antigen retrieval was completed for all antibodies by autoclave. All sections were then immersed in 3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. The primary antibodies of anti-cortactin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), anti-cortactin p421, and anti-cortactin p466 antibodies were both obtained from GenScript (Piscataway, NJ). The sections were incubated overnight with anti-cortactin antibody (1:100) and anti-cortactin p421 (1:60) and p466 antibody (1:60) at 4°C. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide for 30 minutes. They were then treated with peroxidase using the labeled polymer method with DAKO EnVision+ peroxidase (Dako, Carpinteria, CA) for 60 minutes. The peroxidase reaction was visualized using the liquid DAB substrate kit (Zymed Laboratories, San Francisco, CA), and the sections were counterstained with hematoxylin. A consecutive section from each sample processed without the primary antibody was used as a negative control. As the positive control, breast cancer was used according to the data sheet of the antibody provided by the manufacturer. The expression was quantified using the immunoreactivity score (IRS) system: IRS = staining intensity $\times$ percentage of positive cells. Staining intensity was determined as 0, negative; 1, weak; 2, moderate; and 3, strong. The percentage of positive cells was defined as 0, negative; 1, 1%-20%; 2, 21%-50%; and 3, 51%-100% of the cells. In addition, for statistical analysis, the patients were divided into 2 groups according to the IRS value (negative vs positive). The positive group represented those with an IRS greater than the median and the negative group those with staining at or less than the median. In all cases, semiquantitative analyses were performed in $\geq$500 cancer cells and $\geq$200 high-power fields. All evaluations were conducted using a Nikon E-400 microscope, and the digital images were captured (Nikon DU100, Tokyo, Japan). In addition, we used a computer-aided image analysis system (Win ROOF, version 5.0, Mitani, Tokyo, Japan) to calculate the various statistical variables. The slides were examined by 2 of us (Y.M. and T.M.) who were unaware of the clinical features of the patients.

The data are expressed as the mean $\pm$ SD. Student's t test was used for analysis of continuous variables. The chi-square test and Fisher's exact test were used for categorical comparisons of the data. The Scheffé test was used for multiple comparisons of the data. Spearman's correlation coefficient was used to determine the association between 2 continuous variables.

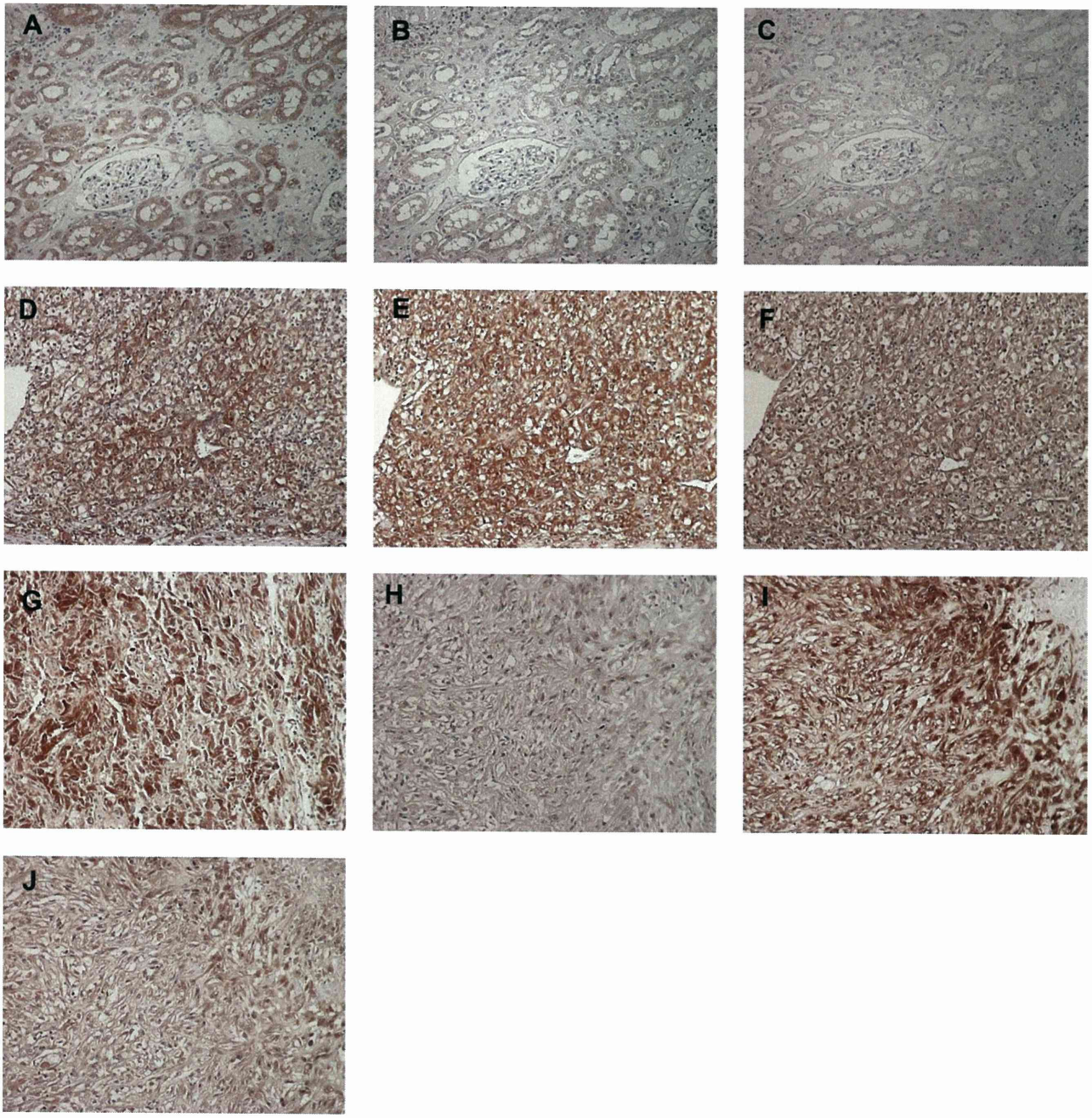

Figure 1. Representative examples of immunohistochemical expression of cortactin, pY421 cortactin, and pY466 cortactin in human CRCC and SRCC. All immunoreactivities were detected in cell cytoplasm. **(A)** In normal renal tubules, moderate and strong expression of cortactin detected. **(B,C)** However, no such expression for cY421 cortactin or cY466 cortactin was evident in same tissues. In CRCC tissue, no remarkable difference found in **(D)** cortactin, **(E)** pY421 cortactin, and **(F)** pY466 cortactin expression. Moderate and strong expression of cortactin detected in **(G)** SRCC. In contrast, some specimens showed **(I)** stronger pY421 cortactin expression in SRCC than in **(H)** cortactin or **(J)** pY466 cortactin (all magnification, ×200).

Survival analysis was evaluated using the Kaplan-Meier analysis and the log-rank test, and variables that achieved statistical significance ($P < .050$) on univariate analysis were subsequently entered into multivariate analysis using Cox proportional hazards analysis (reported as the odds ratios [OR], 95% confidence intervals [95% CI], and P values). All statistical tests were 2 sided, and significance was defined as $P < .050$. All statistical analyses were performed on a personal computer with the statistical package StatView for Windows, version 5.0 (Abacus Concept, Berkeley, CA).

RESULTS

Representative examples of the expression of cortactin and phosphorylated cortactin (pY421 and pY466) in CRCC and SRCC are shown Figure 1. Immunostaining

Table 1. Clinicopathologic features

Variable	Patients (n)	CRCC (n = 33)	SRCC (n = 31)	*P* Value
Age at surgery (y)	64	60.0 ± 11.4	59.8 ± 11.6	.964
Gender	64			.143
Male		28	21	
Female		5	10	
pT stage (n)				
T1	16	8 (24.2)	8 (25.8)	.056
T2	15	4 (12.1)	11 (35.5)	
T3	26	18 (54.5)	8 (25.8)	
T4	7	3 (9.1)	4 (12.9)	
Low (T1-T2)	31	12 (46.0)	19 (61.2)	.079
High (T3-T4)	33	21 (54.0)	12 (38.7)	
Metastasis (n)				.968
Absent	37	19 (57.6)	18 (58.1)	
Present	27	14 (43.4)	13 (41.9)	

CRCC, conventional renal cell carcinoma; SRCC, sarcomatous renal cell carcinoma.

Table 2. Correlation with pathologic features

	CRCC			SRCC		
Variable	Cortactin	pY421	pY466	Cortactin	pY421	pY466
pT stage						
Low (T1-T2)	4.9 ± 2.3	2.3 ± 1.5	3.5 ± 2.6	5.2 ± 2.6	4.8 ± 2.4	2.1 ± 1.7
High (T3-T4)	4.5 ± 1.8	3.4 ± 1.8	2.6 ± 1.8	5.1 ± 2.0	7.8 ± 2.2*	3.4 ± 1.6†
Metastasis						
Absent	4.9 ± 2.1	2.6 ± 1.7	3.3 ± 2.3	5.3 ± 2.7	4.4 ± 2.4	2.5 ± 1.9
Present	4.3 ± 1.9	3.4 ± 1.8	2.5 ± 1.7	5.5 ± 1.9	7.7 ± 2.1*	3.8 ± 1.7

Abbreviations as in Table 1.
* $P < .001$.
† $P = .043$.

of these factors was detected mainly in the cell cytoplasm. Moderate expression of cortactin was found in the normal renal tubules in almost all specimens (Fig. 1A). In contrast, although weak staining for pY421 cortactin and pY466 cortactin was detected in some normal tubules, moderate or strong expression was rare (Fig. 1B,C). With regard to CRCC, the mean ± SD IRSs of cortactin, pY421 cortactin, and pY466 cortactin was 4.7 ± 2.0, 3.0 ± 1.8, and 2.9 ± 2.1, respectively. No significant differences were found in the distribution or pattern of staining of all molecules among the groups (Fig. 1D-F). Representative examples of all molecules in SRCC tissues are shown in Figure 1G-J. Moderate and strong expression of cortactin was often detected in SRCC (Fig. 1G). In contrast, the expression of pY421 cortactin was definitely stronger than that of cortactin and pY466 cortactin in some SRCC tissues (Fig. 1H-J). Finally, the mean ± SD IRS of cortactin, pY421 cortactin, and pY466 cortactin in SRCC was 5.2 ± 2.4, 5.9 ± 2.7, and 2.6 ± 1.8, respectively. No significant differences were found in the cortactin expression ($P = .284$) or pY466 cortactin expression ($P = .505$) between CRCC and SRCC. In contrast, pY421 expression was significantly greater in SRCC ($P < .001$) than in CRCC.

The clinicopathologic features of CRCC and SRCC are listed in Table 1. No significant differences were found between CRCC and SRCC with regard to all the analyzed parameters, including pT stage and metastasis. The relationships among cortactin, pY421 cortactin, and pY466 cortactin expression and pT stage and metastasis are listed in Table 2. With regard to CRCC, the expression levels of all 3 factors did not correlate with pT stage or metastasis. Likewise, cortactin expression was not associated with these parameters in SRCC. However, the IRS of pY421 cortactin in high pT stage disease was significantly greater ($P < .001$) than that in low pT stage disease. In addition, a similar positive relationship was found with metastasis of SRCC ($P < .001$). In contrast, the expression of pY466 cortactin correlated positively with pT stage ($P = .043$) but not with metastasis. To assess the cortactin phosphorylation in CRCC and SRCC, the correlations between the IRSs of cortactin expression and IRSs of phosphorylated cortactin expression were analyzed. For CRCC, positive correlations were found for the expression of both pY421 cortactin ($r = 0.36$, $P < .001$) and pY466 cortactin ($r = 0.51$, $P < .001$). In contrast, for SRCC, no such significant correlations were identified for the expression of pY421 cortactin ($r = 0.15$, $P = .408$) or pY466 cortactin ($r = 0.17$, $P = .359$).

Kaplan-Meier survival curves of cortactin, pY421cortactin, and pY466 cortactin expression are shown in Figure 2A–C. First, cortactin expression was not associated with cause-specific survival (Fig. 2A). Second, patients with SRCC and high expression levels of pY421 cortactin and pY466 cortactin had worse cause-specific survival rates

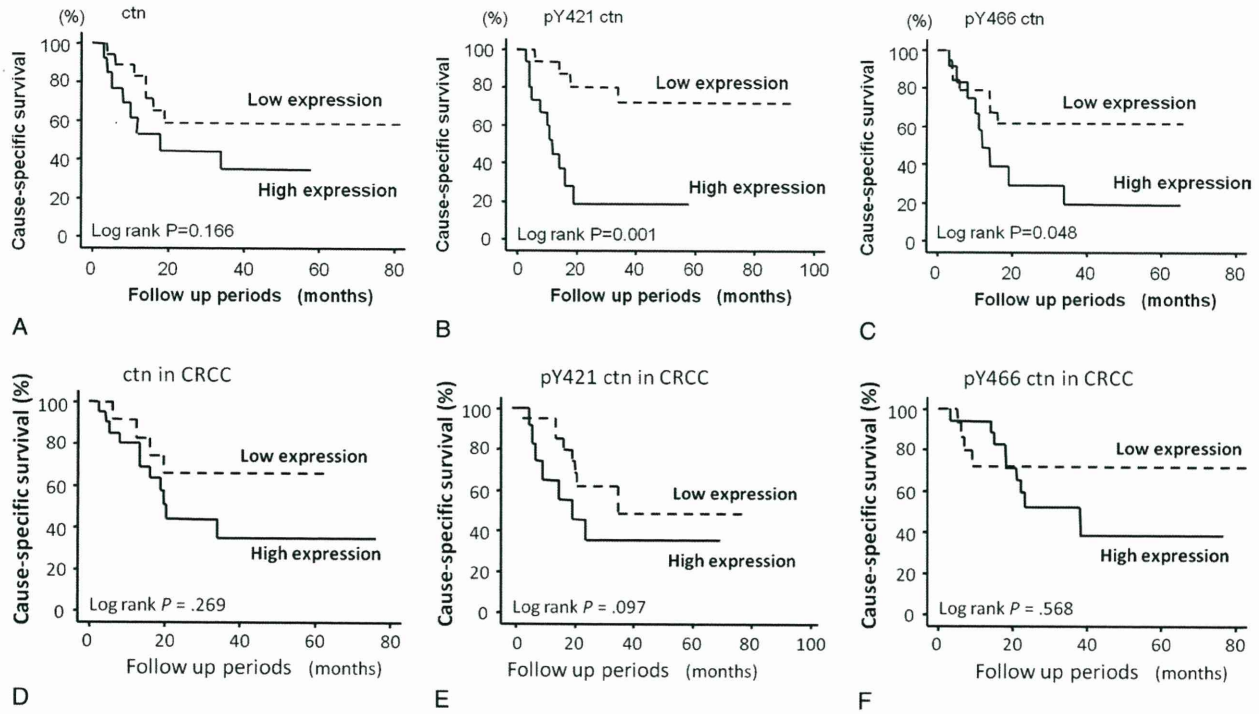

Figure 2. Kaplan-Meier survival curves for cause-specific survival in **(A-C)** SRCC and **(D-F)** CRCC based on expression of **(A,D)** cortactin, **(B,E)** pY421 cortactin, and **(C,F)** pY466 cortactin. Patients with SRCC and high expression of pY421 cortactin had worse survival than those with low pY421 expression. A similar trend was detected for pY466 expression **(C).** In patients with CRCC, none of the parameters was a significant prognostic factor for cause-specific survival.

compared with those with low expression (Fig. 2B,C). Furthermore, high pT stage and the presence of metastasis were significant predictors of cause-specific survival in patients with SRCC (OR 6.25, 95% CI 2.10-18.58, $P < .001$, and OR 4.20, 95% CI 1.41-12.51, $P = .010$, respectively) on univariate analysis. With regard to adjuvant therapy, we speculate that it did not affect the outcome of patients with SRCC or CRCC, because almost all patients received immunotherapy. In contrast, sorafenib was used in only 2 patients with CRCC, and thus no comparison could be made. Finally, a multivariate analysis model that included pT stage and metastasis was conducted to clarify the prognostic role of pY421 cortactin and pY466 cortactin. With regard to pY466 cortactin, its high expression was not a significant prognostic factor (OR 1.54, 95% CI 0.49-4.87, $P = .464$). However, overexpression of pY421 cortactin identified as a significant and independent predictor of cause-specific survival in patients with SRCC (OR 4.53, 95% CI 1.07-19.12, $P = .040$) on multivariate analysis. Figure 2D–F shows the Kaplan-Meier survival curves for cortactin, pY421cortactin, and pY466 cortactin. None of these parameters were associated with cause-specific survival.

COMMENT

The present study is the first report on the clinical significance and prognostic value of cortactin and phosphorylated cortactin in human SRCC tissue. In previous studies, cortactin gene amplification, mRNA expression, and protein expression were investigated in many types of cancer, and high expression levels were found in various cancers, including breast, hepatocellular, head and neck, esophageal, and bladder cancers.[6,8,21-24] With regard to the relationship between cortactin status and clinical features, several studies have reported that high cortactin expression correlates significantly with high pT stage and prognosis in various cancers.[6,16,24] In addition, 1 study reported that suppression of cortactin expression decreased tumor growth in esophageal squamous cell carcinoma cells.[8] In contrast, overexpression of cortactin analyzed using various assays has been reported to promote cell motility and migration in many types of cells.[25,26] Furthermore, small interfering RNA-induced underexpression of cortactin resulted in inhibition of cell motility.[26,27] Thus, there is general agreement that cortactin plays a crucial role in cell invasion in many types of cells, including cancer cells. However, little information is available on the pathologic role of phosphorylated cortactin in vivo, although phosphorylation serves as a major switch and is an essential step in many cell signaling events. One animal experiment study indicated that increased phosphorylation of cortactin in breast cancer cells induced tumor metastasis.[11] However, in contrast to many previous reports, hyperphosphorylated cortactin was reported to inhibit cell motility in gastric cancer and breast cancer cells.[15] Because of these previous findings, we paid special attention to the pathologic roles of cortactin phosphorylation in SRCC. To our knowledge, no

suitable SRCC cell line is yet available. Accordingly, in the present study, we investigated the expression of cortactin and phosphorylated cortactin in human SRCC tissues.

Various sites are known to be tyrosine residues, and phosphorylation of cortactin tyrosine is dependent on various stimulators. For example, Y446 cortactin phosphorylation is induced by protein-tyrosine phosphatase-1B.[28] In addition, Y421, Y466, and Y482 are phosphorylated by various factors, including Src, Ferr, Fyn, Met, and Rac.[13,17-19] In the present study, we investigated the expression of pY421 cortactin and pY466 cortactin, because they are phosphorylated by cancer-related kinases. In addition, a link exists between pY421 cortactin and pY466 cortactin in the regulation of phosphorylation (ie, pY421 cortactin correlates directly with Y466 phosphorylation).[19] Our results have demonstrated that pY421 cortactin expression correlates with tumor progression in patients with SRCC. No such significant association with pY421 cortactin expression was found in patients with CRCC. In addition, pY421 cortactin expression in SRCC was significantly greater than in grade 3-4 or grade 1-2 tumors in CRCC. These results suggest that phosphorylation of Y421 residue is 1 of the important processes to secure the malignant aggressiveness of SRCC and that such a process occurs during sarcomatous changes in RCC cells. A previous study of 124 patients with RCC reported that cortactin expression correlated positively with pT stage and tumor grade.[16] Although our results showed a trend different from that of that report, the results of the 2 studies cannot be compared simply owing to differences in patient background and pathologic type.

With regard to pY466 cortactin, its expression in patients with SRCC and metastasis was greater than in those without metastasis. However, such expression was not associated with pT stage. These results suggest that phosphorylation of Y466 cortactin is, at least in part, an important process in the metastasis of SRCC. In contrast to our expectation, the IRS and clinical significance of pY421 and pY466 expression were different in SRCC. The difference was perplexing; however, another study reported similar findings in gastric cancer. In brief, pY421 cortactin expression was significantly greater in cancer cells than in nontumor (adenoma and non-neoplastic mucosa) cells in gastric cancer tissue.[29] In addition, the same study found significantly lower pY466 cortactin expression in cancer cells than in nontumor cells. Although it was beyond the scope of the present study to discuss the reason for this difference in detail, because tyrosine phosphorylation of cortactin occurs in a progressive manner, with initial phosphorylation at tyrosine 421 followed by 466, some additional factors could be involved in this process. Apart from tyrosine kinases, several serine/threonine kinases and growth factors can either directly or indirectly be associated with the regulation of cortactin phosphorylation.[5] In addition, our results showed that links between cortactin and phosphorylated cortactin in SRCC were different from those in CRCC. On the basis of these results, we speculate that additional factors could influence the regulation of cortactin phosphorylation in SRCC. Additional studies are necessary to understand the exact mechanisms and pathologic significance of cortactin phosphorylation in human cancer tissue.

One of the most interesting results of the present study was that pY421 cortactin expression was independently and closely associated with cause-specific survival in patients with RCC. To our knowledge, little information is available regarding the predictors of survival in patients with SRCC. Admittedly, our study population was too small to allow a firm conclusion regarding the predictive value of pY421 cortactin expression. Additional large studies are needed to dissect the importance of pY421 cortactin expression on survival.

CONCLUSIONS

Our results have showed significantly greater pY421 cortactin expression in SRCC than in CRCC. In addition, the expression levels of pY421 cortactin and pY466 cortactin correlated positively with pT stage, and pY421 cortactin expression also correlated with metastasis. On the basis of these findings, we believe that phosphorylation of cortactin, especially Y421 residue, is a key step in the malignant aggressiveness of SRCC. Our results also suggest that phosphorylation of Y421 residue in cortactin is probably a key step in the malignant transformation of the undifferentiated form of CRCC to SRCC. We speculate that pY421 cortactin expression is a potentially useful predictive factor for cause-specific survival and that inhibition of phosphorylation of cortactin is a potentially useful therapeutic strategy for patients with SRCC.

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