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 $10,000 \times g$ 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).
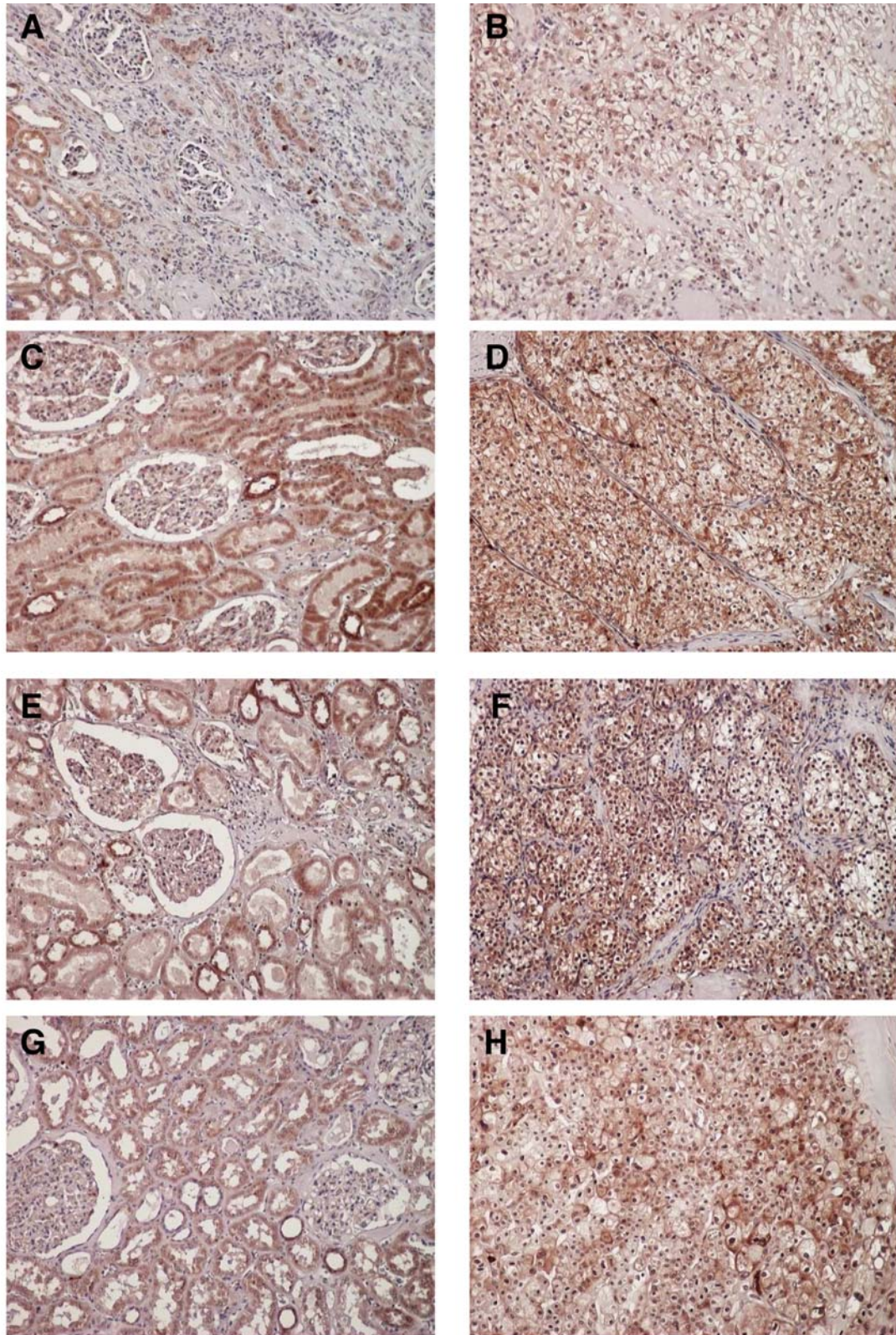
Figure 1. *The expression of each EP receptor was evaluated by immunohistochemistry. EP1R expression in normal renal tubular cells (A) and renal cancer cells (B); EP2R in normal cells (C) and cancer cells (D); EP3R in normal cells (E) and cancer cells (F); EP4R in normal cells (G) and cancer cells (H). EPR immunoreactivities were mainly detected in the cytoplasm. Magnification, ×200.*
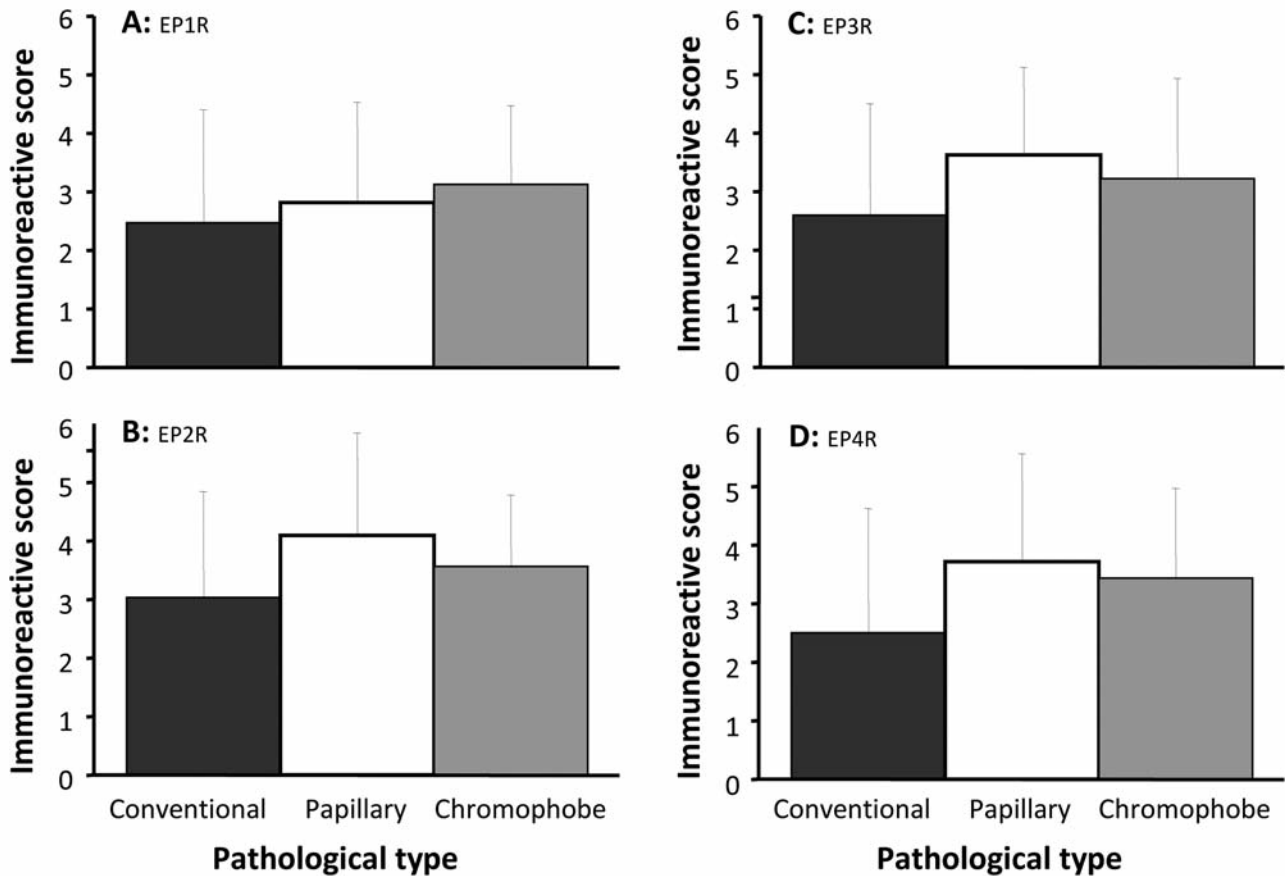
Figure 2. *Relationships among mean (SD) immunoreactivity scores of EPRs and pathological types of renal cell carcinoma. No significant differences were established. Data are mean±SD.*

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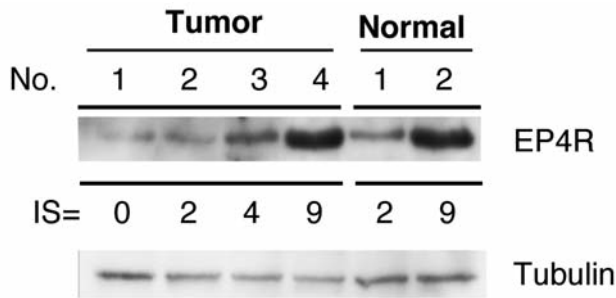
Figure 3. *Representative results of western blot analysis of EP4R expression in normal kidney and renal cell carcinoma tissues. The results were consistent with the determined immunoreactivity scores (IS).*

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

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.
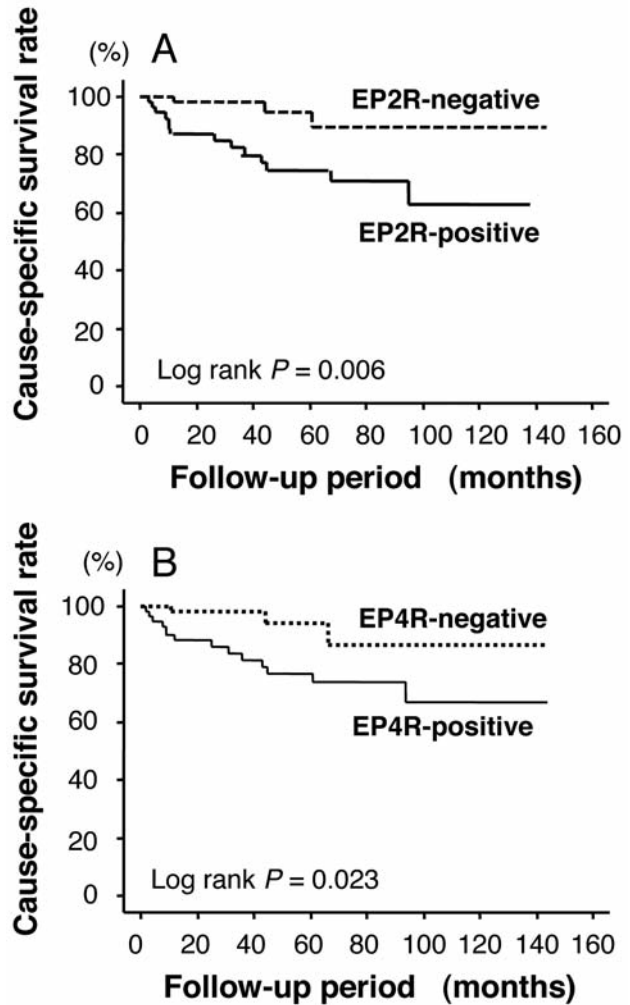
With regard to the relationship between EPR expression and cause-specific survival, the results identified EP2R and EP4R expressions but not EP1R or EP3R as being significant predictors of survival in patients with RCC. Our findings are in accord with previous reports in other cancer types (44, 45). 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.

Conflict of Interest Statement

We declare that we have no conflict of interest.

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