Osteopontin Expression and Its Prognostic Significances in Human Renal Cell Carcinoma

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Background : Osteopontin (OPN) is a glycoprotein and it participates in cell-cell and cell-matrix interactions. *In vitro* studies suggest that the OPN expression is associated with tumor metastasis, and especially with the metastasis of osteotropic tumors originating in breast, prostate and lungs. Since no human tissue study has suggested the means by which OPN participates in the tumorigenesis, angiogenesis, progression and metastasis of renal cell carcinoma (RCC), we evaluated the expression and prognostic significance of OPN in RCC. **Methods :** Immunohistochemistry was performed with using the primary antibody for OPN on the archival paraffin-embedded tissue microarray specimens from 51 RCC patients who underwent radical or simple nephrectomy. **Results :** In the normal kidney specimens, OPN was expressed in a few compressed distal tubules adjacent to the RCCs. In RCCs, the OPN expression was elevated in larger tumors ($p<0.05$) and in the tumor with low microvessel density ($p<0.01$). In the present study, univariate analysis indicated that stage, tumor size, lymph node and distant organ metastasis are significant prognostic factors for disease free survival (DFS) in RCC patients ($p<0.01$), but OPN is not ($p=0.0661$). Multivariate analysis indicated lymph node metastasis is the independent prognostic indicator of DFS ($p<0.05$). **Conclusion :** Though this study has statistical limitations, these results suggest OPN plays a role in tumor progression and metastasis and it may act as a potential prognostic indicator to predict the prognosis of RCC patients.

Key Words : Osteopontin; Carcinoma; Renal cell; Hypoxia; Immunohistochemistry

The stage, tumor size, lymph node metastasis and distant organ metastasis at the initial presentation are the well established prognostic factors for renal cell carcinoma (RCC). Moreover, the last decade has witnessed the additions of various biomolecular variables that are related to metastasis such as the proliferative index, apoptosis, angiogenesis and adhesion molecules. Although these factors provide physicians and researchers with further prognostic clues, the currently available information is insufficient to allow us to assert their usefulness.[1]

Osteopontin (OPN) is a secreted, adhesive glycosylated phosphoprotein, and it was initially described in malignant transformed cells.[2] OPN is normally produced in epithelial cells of the breast, kidney and skin,[3] and it is known to be involved in a number of physiologic and pathologic functions, including angiogenesis and tumor metastasis.[4] OPN is also known to be induced at the protein and mRNA levels by hypoxia via a *ras*-activated enhancer,[5] it increases endothelial cell survival via the $\alpha \upsilon$ integrins[6] and the endothelial motility in the presence of

vascular endothelial growth factor.[7] OPN also engages with several different cellular receptors such as CD44 in combination with the $\alpha \upsilon$- or $\beta 1$- integrins, and it consequently stimulates various signaling pathways to increase cellular adhesion and motility that may ultimately favor tumor progression and metastasis.[8,9] In addition, recent *in vivo* and *in vitro* studies have shown that the serum OPN levels and its expression are elevated in various osteotropic tumors, such as, those of the breast, prostate and lungs,[10,11] although the OPN expression is not elevated in the extra-cellular matrix around tumor cells.[12] Previous human tissue studies based on immunohistochemistry or *in situ* hybridization have suggested that OPN is expressed in the majority of RCC tumor cells.[13] However, it remains unclear how OPN participates in tumor progression or metastasis in RCC patients.

This study examined the OPN expression level under identical conditions in order to make an accurate comparison of tissue microarray (TMA) tumor staining. Furthermore, the relationships between the OPN expression and the clinico-pathological param-

eters with microvessel density (MVD) in RCC patients were analyzed. This study also investigated how OPN is related to angiogenesis under the condition of tumor hypoxia as well as how OPN contributes to tumor progression and metastasis in RCCs.

MATERIALS AND METHODS

Patients

Between January 1999 and December 2000, 54 patients diagnosed with RCC underwent radical or simple nephrectomies at Kyungbook National University Hospital, Daegu, Korea. The clinico-pathological features, including the patients' ages and genders, tumor sizes and stages, histological types, Fuhrman's nuclear grades and clinical outcomes were documented. Two pathologists (Kim TS & Yoon GS) simultaneously reexamined the hematoxylin and eosin stained tissue sections of the primary tumors and reclassified histological types and Fuhrman's nuclear grades. The tumor stages were defined according to the 2002 TNM classification.

Tissue microarray

To construct a TMA, representative tumor tissue sections that were without necrosis, hemorrhage or scirrhous change, were selected from each paraffin block. To minimize the apparent influence of tissue heterogeneity and the effects of technical issues, we took at least two core needle biopsies (3 mm in diameter) from each donor block of RCC tissue and from the adjacent normal renal parenchyma with using a dedicated tissue array instrument (Beecher Instruments, Inc., Sun Prairie, WI). These tissue cores were then arrayed into two empty "recipient" frames to create two similar arrays of 6 by 5 cores. To combine the donor cores, the "recipient" frames were soaked in melted paraffin and heated for 1 h at 58℃.

Immunohistochemistry

After treating the 3 μm TMA sections with microwaves in citrate buffer (0.01 M, pH 6.0), they were immersed in 0.3% H_2O_2 solution and then incubated for 18 h with one of the following primary mouse monoclonal IgG antibodies; anti-human OPN (American Research Products, Inc., Belmont, MA) and anti-human CD31 (DAKO Cytomation, Glostrup, Denmark).

The sections were then incubated in a biotin-streptavidin detection system (Vector Laboratories, Inc., Burlingame, CA) and visualized with 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, MO). Tris-buffered saline (pH 7.0) containing 0.1% Tween-20 was used for each washing step, and all the procedures were performed at room temperature, except for the primary antibody incubation (4℃). Negative control staining was performed by omitting the respective primary antibodies. In this study, three cases were finally excluded from the original file due to insufficient data (n=1) or inadequate material (n=2).

The various immunohistochemical results were scored as follows. The OPN expression was described as the average number of positive cells per 100 cancer cells counted in 5 high power field (HPFs) at ×400. The MVD was determined by counting each vessel in 5 HPFs. If the OPN positive tumor cells were not present in more than 2 HPFs, we considered the sample as OPN negative. Any area showing positive staining for CD31 was counted as a microvessel, whether it possessed a distinct lumen or not, and the large anastomosing sinusoidal vessels were counted as single vessels. For each antibody, the cases were categorized into two groups, e.g., either as being negative or positive for OPN, or as having a low (<mean vessel count/HPF) or high (≥mean vessel count/HPF) MVD.

Statistical analysis

Statistical analysis was performed using Fisher's exact test for categorized variables. Cumulative disease free survivals (DFSs) were estimated using the Kaplan-Meier method, and differences between groups were tested using the log rank test. Cox multiple regression analysis was performed to evaluate the independent predictive values for DFS by performing univariate analysis. A p value of <0.05 was considered to be statistically significant.

RESULTS

Formalin-fixed, paraffin-embedded, archival surgical specimens from 51 RCC patients were assessed. Table 1 compares the OPN expression according to the clinico-pathological data and MVDs of the RCC patients. The mean age of the patients at diagnosis was 54.2 years (range: 29 to 76 years), and 29 men (56.9%) and 22 women (43.1%) were included in the study. Thirty-six patients (70.6%) underwent radical nephrectomy, 14 (27.5%) underwent simple nephrectomy, and one (2.0%) under-

Table 1. Comparisons of clinicopathological data and MVD vs OPN expression in RCC patients

	Patients No. (%)	OPN		p value
		(–)	(+)	
Age (years)		54.2±23.3		
Sex				
M	29 (56.9)	19 (65.5)	10 (34.5)	0.842
F	22 (43.1)	15 (68.2)	7 (31.8)	
TNM stage				
I	38 (74.5)	32 (69.6)	14 (30.4)	0.318
II	8 (17.6)			
III	3 (5.9)	2 (40.0)	3 (60.0)	
IV	2 (3.9)			
T category				
T1a	25 (49.0)	22 (88.0)	3 (12.0)	<0.05
T1b	14 (27.5)	6 (42.9)	8 (57.1)	
T2	9 (17.6)	6 (50.0)	6 (50.0)	
T3b	3 (5.9)			
N category				
N0	48 (94.1)	33 (68.7)	15 (31.3)	0.142
N1	2 (3.9)	1 (33.3)	2 (66.7)	
N2	1 (2.0)			
M category				
M0	50 (98.0)	34 (68.0)	16 (32.0)	0.333
M1	1 (2.0)	0 (0.0)	1 (100)	
Nuclear grade				
1	6 (11.8)	21 (50.0)	10 (50.0)	0.875
2	25 (49.0)			
3	18 (35.3)	12 (66.7)	6 (33.3)	
4	2 (3.9)	1 (50.0)	1 (50.0)	
Histologic type				
Clear cell	40 (78.4)	27 (67.5)	13 (32.5)	0.105
Papillary	2 (3.9)	0 (0.0)	2 (100)	
Chromophobe	9 (17.6)	7 (77.8)	2 (22.2)	
MVD				
<30/ HPF	32 (62.7)	16 (50.0)	16 (50.0)	<0.01
≥30/ HPF	19 (37.3)	18 (94.7)	1 (5.3)	
Follow Up				
NDE	45 (88.2)	32 (71.1)	13 (28.9)	0.087
RDM	6 (11.8)	2 (33.3)	4 (66.7)	

χ^2-test or Fisher's exact test applied; Age was described by mean± 2SD; MVD, microvessel density; NDE, no disease evidence; RDM, recurrence or distant metastasis; No., Number.
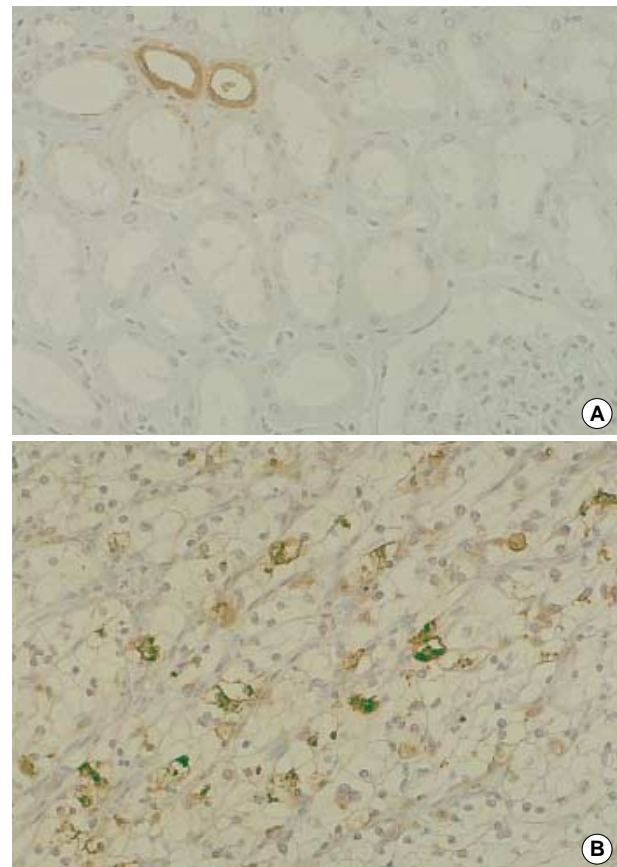
Fig. 1. OPN expression in the cytoplasm of a few distal tubular epithelial cells in the renal cortex adjacent to a RCC capsule (A, ×200), and in tumor cells of clear cell type RCC (B, ×200).

went a renal salvage operation. Thirty-eight (74.5%) patients were classified as having stage I tumors, 8 (17.6%) as stage II tumors, 3 (5.9%) as stage III tumors, and 2 (3.9%) as stage IV tumors. Six tumors were classified as Fuhrman's nuclear grade 1 (11.8%), 25 as grade 2 (49.0%), 18 as grade 3 (35.3%), and 2 tumors as grade 4 (3.9%). Forty tumors were of the clear cell type (78.4%), 2 were of the papillary type (3.9%), and 9 were of the chromophobe type (17.6%). Six patients were finally identified as having the presence of further distant organ metastasis, especially in lung [5 (83.3%)], bone [4 (66.7%)], and etc.

In the control tissue (the normal adjacent kidney tissue), OPN was expressed in the cytoplasm of a few distal tubular epithelial cells and in the renal cortex, which was presumed to be due to the compressive effect of the adjacent RCCs (Fig. 1A). In the RCCs (Fig. 1B, Table 1), the OPN expression was found to be elevated in the tumor cell cytoplasm in accordance with the size of the larger primary tumor (p=0.028) and as the MVD decreased (p<0.001). The OPN expression tended to be elevated in direct relation to the advance in stage, the presence of lymph node or distant organ metastasis at the initial presentation and also to the further distant organ metastasis, but this was without statistical significance (Table 1).

In brief, univariate analysis indicated that stage, tumor size, lymph node metastasis and distant organ metastasis were the independent prognostic factors of DFS (p=0.0010, p=0.0078, p=0.0011 and p=0.0005, respectively). The mean 5-year DFS for the RCC patients decreased as the stage increased [Fig. 2A (65 months vs 30 months, respectively, p=0.0002)]. The mean 5-year DFS of the RCC patients with an OPN expression tended to be less than those without an OPN expression, but again,
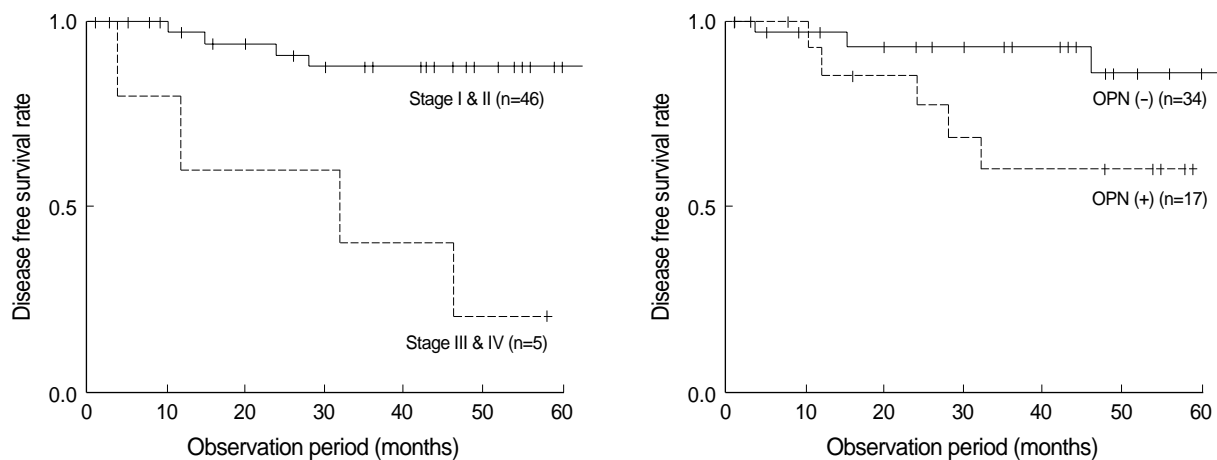
Fig. 2. Kaplan-Meier survival curves for early stages (I and II) and advanced stages (III and IV) (Log-rank test, p=0.0002) RCC patients (A), and OPN negative or positive tumors (Log-rank test, p=0.0661) (B). 5-year mean DFSs for patients with early vs advanced stage RCCs, and OPN negative vs positive RCCs, were 65 vs 34 months, and 66 vs 44 months, respectively.

this was without statistical significance [Fig. 2B (44 months vs 66 months, respectively, p=0.0661)]. Multivariate analysis indicated lymph node metastasis was an independent significant prognostic indicators of DFS.

DFS was defined as the time between surgery and the documented signs (clinical, radiological or pathological) of recurrence or metastasis. The mean duration of follow-up was 34.2 months (range: 1 to 72 months) and 43 patients remained alive without disease recurrence or metastasis at the final follow-up (mean follow-up period: 43 months, range: 1 to 72 months), whereas 6 developed metastatic RCC in lungs and bone (4 out of 6) or soft tissue (1 out of 6) with or without other sites at a mean of 24.3 months (range: 4 to 46 months). The remaining 2 succumbed to death from other causes (mean follow-up period: 26.5 months, range: 1 to 52 months).

DISCUSSION

The tumor stage has been described as the most important single valuable prognostic factor for RCC.[1] Moreover, the patients in the present study tended to present at an earlier stage at the initial diagnosis than those included in a previous multi-center study conducted in South Korea between 1995 to 1997.[14] In the present study, the stage, tumor size, lymph node metastasis and distant organ metastasis were found to be related to patient survival. Moreover, multivariate analysis identified lymph node metastasis (N category) at the initial diagnosis as the significant independent prognostic indicator, which also indicates that the number of RCC patients included in this study was sufficient for statistical purposes.

Oncogenic mutations and hypoxia are both commonly present in solid tumors, especially in actively growing or metastatic tumors.[15] Moreover, mutations in ras are seen in a third of human cancers including RCCs, and transformation with H-*ras* leads to substantially increased or stabilized OPN mRNA levels via the *ras*-responsive element in the OPN promoter.[16,17] In the present study, the OPN expression was inversely related to the MVD, but it was directly related to the T category. Our results suggest that OPN derived from tumor cells under local hypoxic conditions (a low MVD), may act together with other pro-angiogenic molecules such as vascular endothelial growth factor or basic fibroblast growth factor.[18] However, the over-expression of OPN may appear earlier than the angiogenesis induced by these growth factors and the cross-talk to these growth factors.[19,20]

In vivo studies of tumor cells have provided evidences that hypoxia per se induces expression of OPN at the mRNA and protein levels, that proceeds the increased vascular endothelial growth factor mRNA stability.[15,21,22] Others have suggested that OPN increases endothelial cell motility and survival via its interaction with $\alpha\upsilon$ integrins on the endothelial cell surfaces in the presence of vascular endothelial growth factor, via the mediating NF-κB.[6,23] Although we did not observed the OPN expression in the extra-cellular matrix between the tumor cells adjacent to the endothelial cells, the above findings suggest that the OPN expression in RCC tumor cells finally contributes to tumor progression by mediating angiogenesis in a delayed manner.

Recent *in vivo* or *in vitro* studies have suggested that OPN induces extra-cellular matrix invasion by increasing the urokinase type plasminogen activator (u-PA) expression[24,25] or the

matrix metalloproteinase-2 expression.[26] In the present study, the OPN expression tended to be directly related to lymph node or distant organ metastasis at the initial presentation, but this was without significance. Nevertheless, we found that the OPN tended to be over-expressed in the RCC patients with further distant organ metastasis. These results suggest that OPN plays a role for cellular migration and invasion into the extra-cellular matrix, and so it contributes to distant organ metastasis.

It is important to predict an osteo-tropism in cancer metastasis because of its etiologic association with much of the disabling morbidity, including the pain, pathological fractures and hypercalcemia in RCC patients. Previous *in vitro* studies on transformed cells have suggested that the OPN-CD44 interaction serially induces the Rac pathway, which evokes tumor progression and metastasis via cell-cell and cell-matrix interactions.[27] However, it is still unknown why some tumors are predisposed to bone metastasis[28] and it's unclear how OPN plays a role in bone metastasis.[29] In the present study, we found that OPN tends to be over-expressed in the RCC patients with further bone metastasis, but this was without significance. In the present study, we did not examine the OPN serum protein level in the RCC patients or the OPN gene polymorphisms that might have influenced its mRNA or protein levels. However, a recent *in vivo* study demonstrated that transplanted tumors in OPN transgenic mice tended to develop bone metastasis more so than did the OPN knock out mice.[30]

In the present study, we found that the over-expression of OPN is not related to a higher Furman's nuclear grade. The univariate analysis conducted during this study did not identify the Fuhrman's nuclear grade as a significant prognostic factor, despite the fact that this is generally recognized as a significant independent prognostic factor in RCC.[1] Furthermore, seven patients with chromophobe RCC, even with their higher nuclear grade, presented with stage I or II disease and the simultaneous absence of the OPN expression. In addition, both the patients with the papillary type RCC presented with stage I disease and also with the presence of an OPN expression. In the present study, although we were unable to prove whether OPN contributes to the progression of tumor phenotype, our results suggest at least that the OPN expression is related to morphologic trans-differentiation.

Overall, the results suggest that the OPN expression level in RCC tumor cells is directly related to the tumor size and it is inversely proportional to the MVD. In addition, the OPN expression level tends to increase in the RCC patients with lymph node or distant organ metastasis at the initial presentation and it is also increased with further distant organ metastasis. This suggests that the OPN expression is regulated by angiogenesis in the hypoxic state and it may act as a potential indicator for predicting the prognosis of RCC patients.

ACKNOWLEDGEMENTS

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