Expression of CC chemokine receptor 5 in clear cell renal cell carcinoma and its clinical significance

KOO HAN YOO[1], DONG-GI LEE[1], KYU YEOUN WON[2], SUNG-JIG LIM[2], YONG-KOO PARK[2] and SUNG-GOO CHANG[1]

Departments of [1]Urology and [2]Pathology, School of Medicine, Kyung Hee University, Seoul 130-702, Republic of Korea

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Abstract. DNA hypomethylation was the initial epigenetic abnormality recognized in human malignancy. In the present study, the GoldenGate high-throughput genotyping assay was adapted to determine the methylation state of 1,505 specific CpG sites in 807 cancer-related genes. The methylation results revealed that CC chemokine receptor 5 (CCR5) was hypomethylated (mean β-value difference, -0.21) in clear cell renal cell carcinoma (CCRCC) tissue. Tissue samples from 61 CCRCC cases were used for immunohistochemical staining, and patients with low CCR5 expression (n=44) were compared with those with high CCR5 expression (n=17). Tumor (T) stage was significantly lower in the low expression group compared with the high expression group (P=0.047). The Fuhrman grade of patients in the low expression group was significantly lower than that of patients in the high expression group (P=0.044). Whilst the node (N) and metastasis (M) stages of the CCR5 low expression group appeared to be lower compared with those of the CCR5 high expression group; this difference was not statistically significant (N stage, P=0.632; M stage, P=0.896). Additionally, patients in the low expression group had lower risks of postoperative tumor recurrence (P=0.110) and mortality from CCRCC (P=0.159) compared with those in the high expression group, however, this was also without statistical significance. The results indicate that CCR5 hypomethylation is associated with cancer tissue to a greater extent than normal tissue. Although the biological function of CCR5 in CCRCC remains to be established, low CCR5 expression is associated with low T stage and low Fuhrman grade in these patients.

Correspondence to: Professor Sung-Goo Chang, Department of Urology, School of Medicine, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-ku, Seoul 130-702, Republic of Korea
E-mail: sgchang@khu.ac.kr

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Introduction

As the most common neoplasm in the kidney, renal cell carcinoma (RCC) comprises 3% of all cases of adult cancer. In the United States, the incidence of RCC is increasing, with an estimated 63,920 new cases and 13,860 mortalities in 2014 (1). In South Korea, RCC accounts for ~1% of all primary malignancies and is the tenth most common cancer in males (2). RCC may be classified into a number of subtypes, including papillary, chromophobe and collecting duct RCC, clear cell RCC (CCRCC) and other rare subtypes. CCRCC represents 70% of all RCCs and tends to have a poorer prognosis compared with other RCCs (3).

Epigenetic changes may initiate cancer and promote progression (4). Epigenetics may be described as a stable alteration in gene expression potential that takes place during cell proliferation and development, without any change in gene sequence (5). In cancer, DNA hypermethylation is critical in gene expression. Methylation in cytosine-phosphate-guanine (CpG) islands may inhibit gene expression, and CpG hypermethylation in promoter regions may represent one mechanism of carcinogenesis; this may also provide markers for tumor initiation and progression. Hypomethylation is the second type of methylation defect that is observed in a wide range of malignancies. Hypomethylation involves repeated DNA sequences, including long interspersed nuclear elements (LINEs), whereas hypermethylation involves CpG islands.

Chemokines regulate cancer cell migration and contribute to cancer cell proliferation and survival (6). Approximately 45 chemokines and 20 chemokine receptors have been identified, and may be grouped into four categories: C, CC, CXC and CX_3C (7). Chemokine receptors relay their signals through heterotrimeric guanine nucleotide-binding proteins (G proteins) (7). CC chemokine receptor 5 (CCR5) belongs to the trimeric G protein-coupled seven-transmembrane domain receptor superfamily (9), and binds to the RANTES (CCL5), macrophage inflammatory protein (MIP)-1α (CCL3), and MIP-1β (CCL4) chemokines (10). CCR5 is involved in the chemotaxis of leukocytes to sites of inflammation, and is important in the recruitment of macrophages, T cells and monocytes (11). In the present study, the methylation profile of CCR5 was investigated in CCRCC. The association between tumoral CCR5 immunohistochemical expression and clinicopathological parameters was also evaluated.

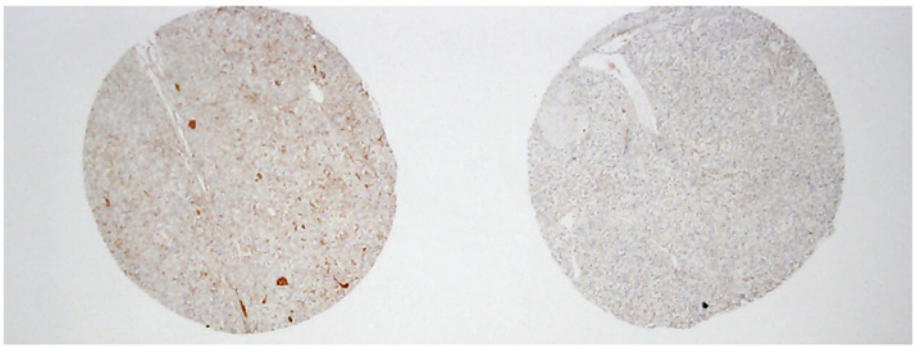

Figure 1. Low magnification view showing high CCR5 expression in the core of clear renal cell carcinoma on the left and low CCR5 expression in the core on the right (magnification, x20). CCR5, CC chemokine receptor 5.

Materials and methods

Patients and preparation of DNA samples. The GoldenGate high-throughput genotyping assay was adapted to determine the methylation state of 1,505 specific CpG sites in 807 cancer-related genes (12). Tissue specimens consisted of 62 cancer tissues and 62 matched adjacent normal tissues from CCRCC patients at Kyung Hee University Hospital (Seoul, South Korea). The Institutional Review Board of Kyung Hee University Hospital approved this study (KHNMC IRB 2013-040). DNA was extracted as previously described (13).

Methylation profiling and validation. Bisulfite conversion of all DNA samples was performed with the EZ-96 DNA Methylation™ kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Following bisulfite treatment, the methylcytosine content was quantified using Illumina's GoldenGate Methylation Cancer Panel I microarray (Illumina Inc., San Diego, CA, USA) (12). The raw methylation ratios were calculated using the Methylation Module in Illumina's BeadStudio following normalization to a background level derived by averaging the signals of an internal negative control. The methylation status of the CpG sites was examined by bisulfite sequencing. The procedure described previously by Herman *et al* (14) was adopted, with slight modification.

Tissue microarray and immunohistochemistry. For immunohistochemical staining, tissue samples from 61 CCRCC cases were used. All neoplasms were surgically resected at Kyung Hee University Hospital between 2006 and 2013. The tissue microarrays were assembled using a commercially available manual tissue microarrayer (Quick-Ray; Unitma Co., Ltd., Seoul, South Korea). Three representative tumor cores with diameters of 2.0 mm were punched from each tumor tissue block. Each of the tissue microarray blocks contained three normal kidney tissue cores (Fig. 1). Immunohistochemistry was performed on 4-μm tissue sections from each tissue microarray block using the Bond Polymer Intense Detection System (Vision BioSystems, Victoria, Australia). Sections were incubated for 15 min at ambient temperature with primary rabbit polyclonal antibodies to CCR5 (dilution, 1:100; Novus Biologicals, Cambridge, UK), using a biotin-free polymeric horseradish peroxidase-linked antibody conjugate system in a Bond-max automatic slide stainer (Vision BioSystems). Nuclei were counterstained with hematoxylin. The negative control was treated in an identical manner using mouse immunoglobulin G instead of primary antibody. The degree of expression based on immunohistochemistry was classified by three pathologists. Semiquantitative analysis of immunoreactivity was performed according to intensity and proportion: The intensity score was as follows: 0, no staining; 1, weak but detectable staining; 2, distinct staining; or 3, strong staining. The proportion score was as follows: 0, 0% stained cells; 1, 1-33% stained cells; 2, 34-66% stained cells; or 3, 67-100% stained cells. These two scores were multiplied together for a total score, categorized as follows: 0-4, low expression; and 5-9, high expression (Fig. 2).

Statistical analysis. Statistical analyses were performed using SPSS software (version 15.0; SPSS, Inc., Chicago, IL, USA). A χ^2 test and linear-by-linear association were used to evaluate the association of the degree of expression by immunohistochemistry with clinicopathological variables. The overall survival was defined as the time interval between the primary radical or partial nephrectomy and the last follow-up or mortality. The recurrence-free survival period was defined as the time interval between the primary radical or partial nephrectomy and the last follow-up or evidence of recurrence. Survival was estimated using the Kaplan-Meier method. All statistical tests were two sided, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Methylation status of the CCR5 gene in CCRCC. The methylation status of CCR5 in the 62 cancer tissues and 62 matched adjacent normal tissues was analyzed using a GoldenGate high-throughput genotyping assay. The methylation status is represented by the β-value (15). The results revealed that the mean β-values for CCR5 were 0.44 in the normal tissues and 0.23 in the CCRCC tissues; the mean β-value difference was -0.21. The methylation status of the CpG sites was examined by bisulfite sequencing, revealing that CCR5 hypomethylation occurred to a greater extent in the cancer tissues than the normal tissues. Methylation profiling revealed ~10 significant genes, including CCR5. However, the other genes revealed no significant results using immunohistochemical staining.

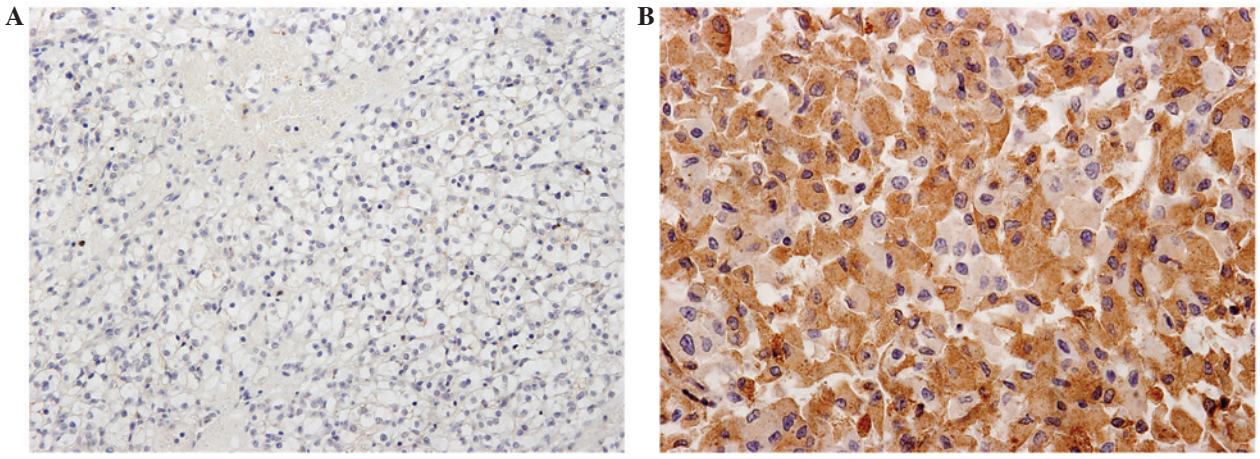

Figure 2. (A) High magnification view showing no cytoplasmic CCR5 expression in CCRCC cells (magnification, x200). (B) High magnification view showing diffusely and strongly cytoplasmic CCR5 expression in CCRCC cells (magnification, x400). CCR5, CC chemokine receptor 5; CCRCC, clear cell renal cell carcinoma.

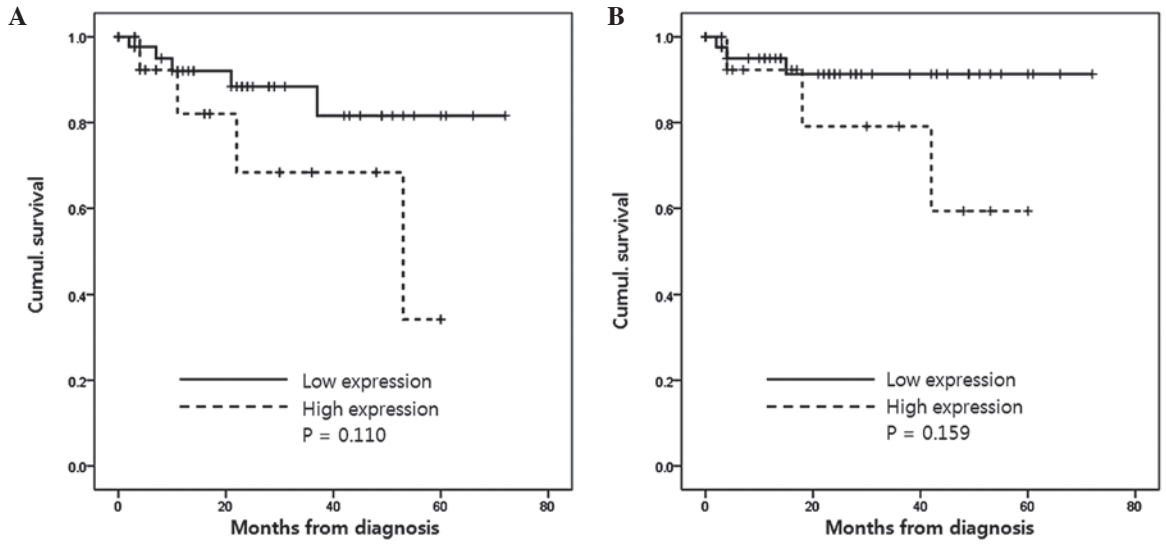

Figure 3. (A) Recurrence-free survival and (B) overall survival Kaplan-Meier curves in clear cell renal cell carcinoma patients.

CCR5 expression in CCRCC. Normal glomeruli and tubules exhibited no immunoreactivity for CCR5. However, 17 cases out of a total of 61 CCRCCs exhibited high CCR5 expression. CCR5 was strongly expressed in the cytoplasm of CCRCC cells.

Association between CCR5 expression and clinicopathological parameters. Patients with low CCR5 expression (n=44) were compared with those with high expression (n=17). The low expression group was composed of 28 males and 16 females, while 12 males and 5 females formed the high expression group (P=0.766). The mean age (± standard deviation) was 61.75±8.35 years in the low expression group and 56.74±18.91 years in the high expression group (P=0.156). The tumor (T) stage was significantly lower in the low CCR5 expression group compared with the high expression group (P=0.047). In addition, the low expression group was associated with a significantly lower Fuhrman grade compared with that of the high expression group (P=0.044). Although the node (N) stage and metastasis (M) stage of the low CCR5 expression group were lower than those of the high expression group, this difference was not statistically significant (N stage, P=0.632; M stage; P=0.896; Table I). The low expression group also had lower risks of post-operative tumor recurrence (P=0.110) and mortality from CCRCC (P=0.159), however, these results were not statistically significant (Fig. 3).

Discussion

DNA hypomethylation was the first epigenetic abnormality recognized in human malignancy (16). However, the hypermethylation of promoters of tumor-suppressor genes is focused in carcinogenesis (17). Recent high-resolution genome-wide studies confirm that DNA hypomethylation frequently co-occurs with hypermethylation of the genome in cancer. DNA hypomethylation may be detected early in carcinogenesis, however, it is also often associated with cancer progression (18). Hypomethylation may promote carcinogenesis by causing an increase in DNA recombination, and via direct and indirect effects on protein expression (19). Repeated DNA sequences that are frequently hypomethylated in cancer tissue may act as tumor markers for cancer diagnosis and

Table I. Association between CCR5 expression and clinicopathological characteristics in clear cell renal cell carcinoma.

	CCR5, n		
Clinicopathological variable	Low expression	High expression	P-value
Patients	44	17	
Gender			0.766
Male	28	12	
Female	16	5	
Fuhrman nuclear grade			0.044
I	1	0	
II	23	4	
III	17	11	
IV	3	2	
T stage			0.047
T1	36	9	
T2	2	3	
T3	6	5	
N stage			0.632
N0	42	16	
N1	2	1	
M stage			0.896
M0	41	16	
M1	3	1	

CCR5, CC chemokine receptor 5; T, tumor; N, node; M, metastasis.

prognosis (17). Hypomethylated DNA sequences are more sensitive markers than unique sequences that are subject to cancer-linked DNA hypermethylation.

DNA hypomethylation is a common methylation defect that is observed in a wide variety of malignancies (20); it is common in solid tumors, including prostate, cervical and metastatic hepatocellular cancer (21-23). Global hypomethylation, such as in breast, cervical and brain cancer, has been shown to progressively increase with grade of malignancy (17). Patients with immunodeficiency, centromeric instability or facial abnormalities, as well as numerous other malignancies, exhibit severely hypomethylated pericentric heterochromatin regions on chromosomes 1 and 16 (24). DNA hypomethylation has been hypothesized to contribute to oncogenesis by the activation of oncogenes, by the activation of latent retrotransposons or by chromosome instability (25).

Jürgens *et al* (26) reported an association between hypomethylation and urothelial carcinoma. In the study, DNA methylation alterations were analyzed in 6 urothelial carcinoma cell lines and 13 tumor tissues. L1 LINE sequence methylation was reduced in the majority of tumors compared with that of normal bladder mucosa. DNA hypermethylation of the calcitonin gene CpG islands was restricted to the cell lines and was not detected in the primary tumor tissues. L1 LINE hypomethylation appears to be frequent in urothelial carcinoma and may be useful for diagnostic or prognostic purposes.

A number of studies have previously investigated CCR5 expression and solid tumor carcinogenesis. A study by Zimmermann *et al* (27) revealed that a low expression level of CCR5 in human colorectal cancer is associated with lymphatic dissemination and reduced $CD8^+$ T-cell infiltration. CCR5 expression that was weak or absent was also significantly associated with lymph node metastasis and advanced stage. The study hypothesized that T-cell retention at the tumor site may be mediated by CCR5-dependent mechanisms of immune and tumor cells, and concluded that CCR5 may play a role during the progression of colorectal carcinoma, possibly preventing cancer progression. However, van Deventer *et al* (28) revealed that CCR5 expression in stromal, and not hematopoietic cells, contributes to tumor metastasis. The study reported that mice expressing CCR5 exhibited enhanced local tumor growth and an impaired response to vaccine therapy compared with CCR5-knockout mice. Lin *et al* (29) reported that CCR5 and CCL5 were highly expressed in breast cancer lymph node metastasis, and that CCR5-CCL5 interaction promotes cancer cell migration under hypoxic conditions.

In the present study, the methylation profile of CCR5 in CCRCC, and the association between tumoral CCR5 immunohistochemical expression and clinicopathological parameters were investigated. Patients with low CCR5 expression were compared with those with high CCR5 expression, and the results revealed that T stage was significantly lower in the low expression group compared with the high expression group. The Fuhrman grades of the low expression group were reduced compared with those of the high expression group. The low CCR5 expression group tended to be of lower N and M stages

compared with the high expression group, and the low expression group also tended to have lower risks of post-operative tumor recurrence and mortality from CCRCC, however, these differences were not statistically significant.

In summary, the present study indicates that the CCR5 gene is hypomethylated to a greater extent in cancer tissue compared with in normal tissue. Although the biological function of CCR5 in CCRCC remains unclear, the CCRCC patients with low CCR5 expression exhibit a low T stage and low Fuhrman grade, however, this is not statistically significant. Determining the expression of mRNA in tumor cells is required as this may aid in determining the diagnosis and prognosis of CCRCC cases. This study reveals CCR5 as a potential new tumor marker for kidney cancer.

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