DNA hypomethylation of interleukin 8 in clear cell renal cell carcinoma

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Abstract. DNA hypomethylation is associated with carcinogenesis due to its involvement in cancer initiation and progression. In this study we analyzed the hypomethylation status of candidate genes in clear cell renal cell carcinoma (CCRCC) using a large-scale, high-throughput DNA methylation profiling technique. Results revealed that hypomethylated interleukin 8 (IL8) gene was the most prominent of the candidate genes, with a beta value difference of 0.406 (cancer tissue mean beta value, 0.346; normal tissue mean beta value, 0.752). Validation results using sequencing analysis demonstrated that the methylation rate was 2.4% in cancer tissue and 14.7% in normal tissue, whilst the non-methylation rate was 82.9% in cancer tissue and 52.9% in normal tissue. However, the hypomethylation status of IL8 had no significant relationship with Fuhrman's nuclear grade, tumor node metastasis (TNM) stage or survival (P>0.05). We revealed that the IL8 gene is maximally hypomethylated in cancer tissue compared to normal tissue.

Introduction

Interleukin 8 (IL8) is produced by macrophages, neutrophils, endothelial cells and cancer cells. It is a proinflammatory chemokine associated with the promotion of neutrophil chemotaxis and degranulation (1). This chemokine activates intracellular signaling pathways using G protein-coupled receptors (1). A number of chemokines, such as IL8, promote and regulate neoplastic progression, including angiogenesis and metastasis (2). IL8 is a potent angiogenic factor and controls the expression of vascular endothelial growth factor (VEGF) in endothelial cells (3). Overexpression of VEGF is closely associated with the progression of clear cell renal cell carcinoma (CCRCC) (4).

Hypomethylation of genomic DNA has been associated with increased rates of genomic instability (5). Hypomethylation of CpG dinucleotides in genomic DNA is known as one of the somatic epigenetic alterations identified in human cancers (5). DNA hypomethylation is postulated to affect carcinogenesis due to its involvement in cancer initiation and progression (6). In this study, we analyzed the hypomethylation status of candidate genes, including IL8, in CCRCC using a large-scale, high-throughput DNA methylation profiling technique. The correlation between the hypomethylation status of the candidate gene (IL8) and clinicopathological parameters was examined.

Material and methods

Tissue specimens consisted of 46 cancer tissues and 46 matched adjacent normal tissues from CCRCC patients who had undergone nephrectomy at Kyung Hee University Hospital between 1999 and 2005. The study was approved by the institutional review board at Kyung Hee University Medical Center. Preparation of DNA samples and DNA extraction were implemented as previously described (7). Bisulfite conversion of all DNA samples was performed with EZ-96 DNA Methylation kit (Zymo Research, Orange, CA, USA). The methylcytosine content was quantified using a GoldenGate Methylation Cancer Panel I microarray (Illumina, San Diego, CA, USA) (8). Following the bisulfite conversion and methylation chip assay, technical replicates were prepared for each sample using the same converted DNA. Beta values for matching normal and cancer clinical samples were compared; differential methylation was readily detected (8). The methylation status of CpG sites was examined by bisulfite sequencing according to the protocol employed by Herman *et al* (9). The methylation status of the 1505 CpG sites was measured using a data matrix of beta values. An additional filter based on a Student's t-test was applied. This required a maximal difference in beta value between the two groups (10). The hypomethylation status and clinicopathological characteristics of CCRCC patients were analyzed.

Results

We identified one marker (IL8) out of 807 cancer-related candidate genes (Fig. 1). Results revealed that the mean beta value difference of IL8 was 0.406 (cancer tissue mean beta

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Table I. Baseline characteristic of clear cell renal cell carcinoma (CCRCC) patients.

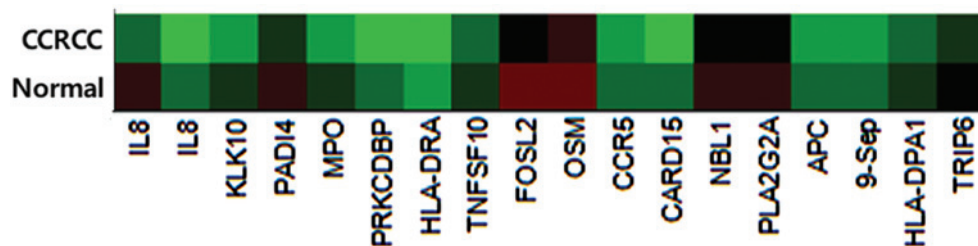|	Weak hypomethylation (n=23)	Strong hypomethylation (n=23)	P-value
Age	61.30±11.166	62.74±10.618	0.502
Gender			0.738
Male	18	16	
Female	5	7	
Fuhrman's nuclear grade			
I	2	2	
II	11	7	
III	7	9	
IV	3	5	0.316
TNM stage			
T1	16	15	
T2	4	4	
T3	3	4	
T4	0	0	0.695
N0	22	22	
N1	1	1	1.000
M0	21	19	
M1	2	4	0.386

Figure 1. Heat map analysis of clear cell renal cell carcinoma (CCRCC) and normal samples. Green and red colors represent low and high methylation levels, respectively. Of 807 cancer related genes, interleukin 8 (IL8) had the largest beta value difference (-0.406) between cancer and normal tissue.
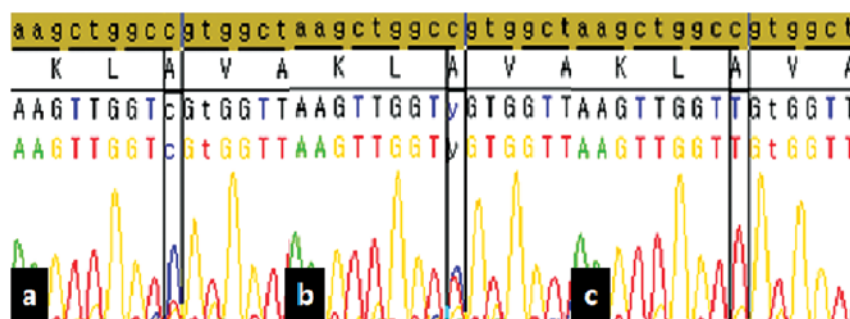

Figure 2. Sequencing analysis of abnormal methylation in the promoter region of interleukin 8 (IL8). (a) Methylated CpG islands in the cancer portion; the Cs of the CpG islands remain unchanged after modification by sodium bisulfite. (b) Heterogenous CpG islands; mixed methylated and unmethylated CpG islands. (c) Unmethylated CpG islands in which Cs become Ts following modification with sodium bisulfite.

value, 0.346; normal tissue mean beta value, 0.752). Of the 46 CCRCC patients, two groups were defined according to their beta value difference; weak hypomethylation (a difference smaller than the mean value) and strong hypomethylation (a difference greater than the mean value). Table I indicates that the hypomethylation status of IL8 had no significant correlation with either Fuhrman's nuclear grade or tumor node metastasis (TNM) stage (P>0.05).
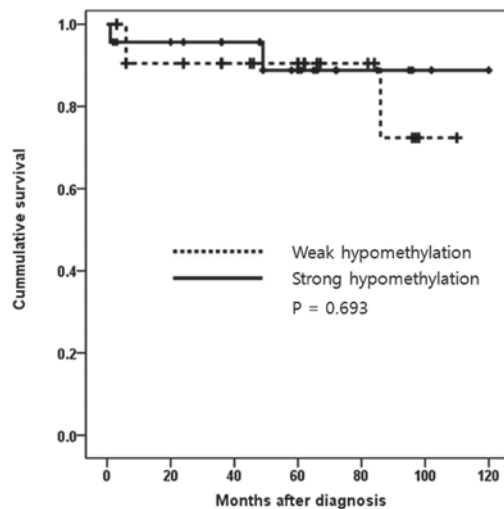
Figure 3. Kaplan-Meier curves of overall survival in clear cell renal cell carcinoma (CCRCC) patients by interleukin 8 (IL8) methylation status.

We examined the methylation status of the CpG site by bisulfite sequencing. Fig. 2 reveals the hypomethylation results of IL8 using bisulfite sequencing. Validation results using sequencing analysis demonstrated that the methylation rate was 2.4% in cancer tissue and 14.7% in normal tissue, whilst the non-nmethylation rate was 82.9% in cancer tissue and 52.9% in normal tissue. The overall survival time of CCRCC patients was analyzed according to either a weak or strong hypomethylation status of IL8 (Fig. 3). There was no statistical influence of IL8 hypomethylation status on survival, as demonstrated by the Kaplan-Meier survival curve (P=0.693).

Discussion

In this study, beta value differences of cancer and normal tissue were not associated with clinicopathological status. However, DNA hypomethylation of IL8 was widely detected in cancer compared to normal kidney tissue. Alteration of genomic DNA methylation, such as hyper- and hypomethylation, affects human tumorigenesis (5). Hypermethylation of promoter CpG islands is capable of inactivating important tumor-suppressor genes (11). Hypomethylation of genomic DNA has been associated with increased rates of genomic instability (6,12).

Suzuki *et al* demonstrated that genomic DNA hyper- and hypomethylation alterations in gastrointestinal cancer were distributed gradually and increased with cancer patient age (13). It was concluded that age-dependent accumulation of DNA demethylation precedes diploidy loss in gastrointestinal cancer. Cadieux *et al* demonstrated that hypomethylation of Sat2 sequences is associated with copy number alterations of the adjacent euchromatin in human glioblastomas (14). This implicated hypomethylation to be a predisposing factor for specific genetic alterations in glioblastoma. The analysis between DNA hypomethylation and prostate cancer was reported by Yegnasubramanian *et al*, who performed a tiered gene expression microarray and bisulfite genomic sequencing-based approach to identify the methyla-

tion status of prostate cancer (5). The authors demonstrated that a class of cancer testis antigen genes undergoes CpG island hypomethylation and overexpression in primary and metastatic prostate cancer. DNA hypomethylation patterns were heterogenous across different metastatic sites within the same patients.

IL8 is a proinflammatory cytokine for leukocytes and is involved in tumor growth, metastasis and survival of solid organ cancer (15,16). Certain studies have analyzed IL8 methylation status in human cancer (17,18). Dimberg *et al* revealed the protein expression of IL8 in plasma, tumor and paired normal tissue, and the methylation status of the IL8 gene in colorectal cancer (17). The authors demonstrated that a significantly higher level of IL8 was present in cancer tissue compared to normal tissue. IL8 hypomethylation was detected in 64% of the cancer tissue, whereas no hypomethylation was found in the paired normal tissue. De Larco *et al* analyzed the correlation between the metastatic potential of breast carcinoma cell lines and the ectopic expression of IL8 (18). The authors revealed that an aberrant methylation pattern may be responsible for the differences in IL8 between high and low metastatic cell lines. Two CpG sites of IL8 were fully methylated in the high metastatic cell lines. However, these cell lines produced large quantities of IL8. The authors suggested that there may be additional epigenetic control mechanisms that have not yet been fully appreciated or explored.

In summary, this study demonstrated that the IL8 gene was maximally hypomethylated in CCRCC cancer tissue compared to normal tissue. However, levels of DNA hypomethylation were not correlated with the clinicopathological status of the patient.

Acknowledgements

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