Aberrant Methylation of the *Vimentin* Gene in Hepatocellular Carcinoma

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Abstract. *Background: Recently, it was shown that the Vimentin gene, usually activated in mesenchymal cells, was highly methylated in colorectal carcinoma. Materials and Methods: The methylation status of the Vimentin gene was examined in primary carcinomas and the corresponding normal tissues derived from 43 patients with hepatocellular carcinoma (HCC) using quantitative methylation-specific PCR (qMSP) and the correlation between the methylation status and the clinicopathological findings was evaluated. Results: Aberrant methylation of the Vimentin gene was detected in 24 out of the 43 (56%) primary HCC. This result suggested that the aberrant methylation of the Vimentin gene was frequent in HCC. Subsequently, clinicopathological data were correlated with the methylation status. A significant difference was observed in the value of alpha-fetoprotein (AFP) (p=0.045), maximal tumor size (p=0.048) and TNM stage (p=0.043) between the methylation-positive and -negative cases. Conclusion: Aberrant methylation of Vimetin might be an early event in the course of hepatocarcinogenesis.*

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors worldwide and the third leading cause of cancer death, next to lung and stomach cancer (1). To eliminate this fatal cancer, a variety of treatments are employed, including surgical operations, percutaneous ablation, transcatheter arterial embolization and chemotherapy. However, the prognosis of patients with HCC remains poor. For the improvement of that prognosis, it is critical to identifiy markers of malignancy of this cancer.

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Key Words: Vimentin, quantitative methylation-specific PCR, hepatocellular carcinoma.

Aberrant methylation of CpG-rich sequences (CpG islands) is an epigenetic change that is common in human cancer, including HCC (2). To date, approximately 94 genes have been found to be hypermethylated in HCC (3-9). Multistep hepatocarcinogenesis has also been demonstrated to include the inactivation of adenomatous polyposis coli (*APC*), glutathione S-transferase P1 (*GSTP1*), Ras association (RalGDS/AF-6) domain family 1A (*RASSF1A*), cyclooxygenase-2 (*COX-2*), *E-cadherin* and *p16* by promoter hypermethylation (10). There has been substantial interest in attempting to adapt such cancer-associated aberrant gene methylation for clinical use.

Recently, it was shown that the *Vimentin* gene, usually activated in mesenchymal cells, was highly methylated in colorectal carcinoma (11). Several reports have indicated *Vimentin* methylation was detected in 53-84% of colorectal carcinomas (12-14). Moreover, *Vimentin* methylation can be applied as a diagnostic tool of colorectal carcinoma in a fecal DNA test. These results prompted us to examine the methylation status of the *Vimentin* gene in HCC surgically removed in our hospital and to evaluate the correlation between the methylation status and the clinicopathological findings.

Materials and Methods

Sample collection and DNA preparation. Forty-three primary tumor and background liver tissue specimens were collected consecutively at Showa University Fujigaoka Hospital from HCC patients during hepatectomy. Eleven normal liver tissue specimens were collected as a control from patients with liver-metastatic colorectal cancer during hepatectomy. All the specimens were confirmed histologically. Written informed consent, as required by the Institutional Review Board, was obtained from all the patients. The collected samples were stored immediately at –80˚C until analysis. DNA was prepared as described elsewhere (15). The clinicopathological profiles of the patients enrolled in the study are shown in Table I.

Sodium bisulfate modification. One μg of the genomic DNA extracted from the HCC specimens was subjected to bisulfite treatment using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany) as described elsewhere (16).

Table I. *Clinicopathological features and Vimentin methylation in HCC.*

Clinicopathological feature	Variable	No. of cases	Vimentin methylation		*p*-Value
			+	–	
Gender	Male	32	20	12	0.13[a]
	Female	11	4	7	
Age (years, mean±S.D.)		43	68.2±7.9	70.5±8.2	0.18[b]
Maximal tumor size (mm, mean±S.D.)		43	27.6±13.6	36.5±21.4	0.048[b]
AFP (ng/ml, mean±S.D.)		43	307.7±611.2	1705±3899.3	0.045[b]
PIVKA-II (AU/ml, mean±S.D.)		43	4.2±13.9	4.1±9.7	0.50[b]
Number of tumor per patient		43	1.5±0.7	1.7±1.2	0.18[b]
Virus type	HBV	15	10	5	0.29[a]
	HCV	28	14	14	
Background	LC	21	10	11	0.29[a]
	else	22	14	8	
TNM stage	I, II	40	24	16	0.043[a]
	III, IV	3	0	3	
Total		43	24	19	

[a]Chi-square test; [b]Student's *t*-test; AFP, alpha-fetoprotein; PIVKA-II, protein-induced vitamin K absence or antagonist II; LC, liver cirrhosis; HBV, hepatitis B virus; HCV, hepatitis C virus.

Quantitative methylation-specific polymerase chain reaction (qMSP). The bisulfite-treated DNA was amplified with a qMSP that was conducted in a Thermal Cycler Dice® Real-Time System TP800 (Takara Bio Inc., Otsu, Japan). Thermocycling was carried out in a final volume of 25 μl containing 1.0 μl of the DNA sample, 100 nM each of the *Vimentin* or β-actin primers (forward and reverse), and 12.5 μl of SYBR Premix Ex Taq II (Takara Bio Inc.), which consisted of a Taq DNA polymerase, reaction buffer and deoxynucleotide triphosphate mixture. The qPCR primer sequences for *Vimentin* have been described elsewhere (11) and were: *Vimentin* MS (sense), 5'-TCGTTTCGAGGTTTT CGCGTTAGAGAC-3', and *Vimentin* MAS (antisense), 5'-CGACTAAAACTCGACCGACTCGCGA-3'. The PCR amplification consisted of 40 cycles (95˚C for 5 s and 55˚C for 30 s) after an initial denaturation step (95˚C for 10 s). The bisulfite-treated DNA obtained from L132 cell line cells that was fully methylated by *Sss*I methylase was used as a positive control. To correct for differences in both quality and quantity between samples, β-actin was used as an internal control. The targets were obtained from the same bisulfite-treated DNA.

Vimentin methylation scores. The relative levels of *Vimentin* methylated DNA in the HCC that were normalized to the internal control β-actin were calculated. The *Vimentin* methylation score in each tissue was defined as follows: relative level of methylated *Vimentin* in tumor/average relative level of methylated *Vimentin* in normal liver tissue that were collected from patients with liver-metastatic colorectal cancer. *Vimentin* methylation was positive when the methylation score was more than 2.0.

Statistical analysis. The associations between the *Vimentin* methylation and the clinicopathological parameters were analyzed using Chi-square tests or Student's *t*-tests. A p-value <0.05 indicated statistical significance.

Results

Aberrant methylation of *Vimentin* was detected in 24 out of the 43 (56%) primary HCC suggesting that the aberrant methylation of *Vimentin* was frequent in HCC.

Subsequently, the clinicopathological data were tested for correlation with the methylation score. No significant correlations were observed between the presentation of aberrant methylation in the HCC and patient gender, age, tumor marker (PIVKA-II) concentration, the number of primary tumors per patient, virus type or background (Table I). A significant difference was observed in the mean maximal tumor size (p=0.048), tumor marker (AFP) concentration (p=0.045) and TNM stage (I, II *vs.* III, IV) (p=0.043) between the methylation-positive and methylation-negative patients (Table I).

Discussion

Vimentin, a member of the intermediate filament protein family, exhibits a complex pattern of gene expression that can be observed at several levels (17). Vimentin is first expressed during development in mesoderm cells located between the primitive streak and the proximal endoderm. Many tissues differentiate from this origin and continue to

express vimentin. Moreover, it has been suggested that vimentin can act as a signal transducer, relaying information from the extracellular matrix to the nucleus (17).

Recently, it has been suggested that the presence of chronic infection from hepatitis virus or differences in the course of inflammation may reflect the frequency of methylation events, which are finally related to the increasing risk of liver cancer. (18) In the present study, the background liver tissue which was resected with the HCC presented pathologically chronic hepatitis or liver cirrhosis. Aberrant methylation of the *Vimentin* gene was also observed in this inflammatory liver. Interestingly, the relative level of *Vimentin* methylated DNA in the HCC was mostly less than in the background liver tissue. These results suggested that aberrant methylation of *Vimetin* would relate to an early event in the course of hepatocarcinogenesis.

In conclusion, *Vimentin* methylation occurs frequently in HCC. Because of the frequent methylation of the *Vimentin* gene and the high sensitivity of qMSP, it could potentially be used for the monitoring of HCC by the detection of *Vimentin* methylation in clinical samples such as serum (19, 20).

Acknowledgements

We thank M. Ogata for her technical assistance.

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Received January 8, 2011
Revised March 15, 2011
Accepted March 16, 2011