Frequent Promoter Methylation of M-cadherin in Hepatocellular Carcinoma is Associated with Poor Prognosis

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Abstract. *Background: Many cadherins (CDH) are associated with various types of cancer and their genetic and epigenetic alterations might be involved in carcinogenesis. Materials and Methods: We examined the methylation status of CDH genes in primary hepatocellular carcinoma (HCC) and corresponding noncancerous liver tissues derived from 47 patients, and evaluated the correlation with clinicopathological parameters. Results: Hypermethylation was detected at a ratio ranging from 0% to 55.3%. In particular, M-cadherin (CDH15) was the most hypermethylated of 7 CDH genes. Patients with methylated M-cadherin had shorter 5-year survival rates than patients with unmethylated M-cadherin (overall survival rates, 67.4% vs. 82.7%; p=0.0167) when assessed using Kaplan-Meier curves. Multivariate analysis revealed that M-cadherin methylation status was an independent predictor of survival. Conclusion: We found that M-cadherin methylation status has prognostic significance for the poorer survival of patients with HCC. This is the first definitive report of a correlation between M-cadherin and the prognosis of patients with HCC.*

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and is generally associated with a poor prognosis (1). The disease is highly prevalent in Asia but relatively rare in developed countries, although its incidence is increasing in both the United States and Japan (2). In many cases, HCC develops from chronic liver disease due to infection with hepatitis B virus (HBV), or hepatitis C virus (HCV), or exposure to drugs such as aflatoxin B1. Many more studies have shown that multiple genetic alterations are associated with HCC, but the molecular mechanisms of hepatocarcinogenesis are not yet clear.

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Transcriptional silencing of tumor suppressor and mismatch repair genes due to hypermethylation of 5'CpG islands is observed in a variety of malignancies (3, 4). Several reports indicated that tumorigenesis is related to loss of function of a tumor suppressor gene as a result of loss of heterozygosity (LOH), gene mutations, and hypermethylation of promoter regions (5). Notably, allelic imbalance at chromosome 17p13 and frequent mutations of the p53 gene have been correlated with exposure to aflatoxin B1 and chronic HBV infection (6). Mutations of β-catenin or its binding partner axin 1, leading to abnormal activation of the Wnt pathway, have been implicated in about one third of HCC cases (7, 8).

In recent years, there has been increasing interest in a superfamily of transmembrane glycoproteins, the cadherins (CDH). Cadherins are prime mediators of calcium-dependent cell-cell adhesion in normal cells and are also involved in contact inhibition of cell growth by inducing cell cycle arrest (9, 10). Many cadherins are associated with cancer. For example, the epithelial E-cadherin (CDH1), a putative tumor suppressor, is deregulated in HCC, esophageal, gastric, colon, pancreatic, lung, breast and prostate cancer (11-17). In contrast, increased expression of other cadherins such as N-cadherin (CDH2), P-cadherin (CDH3), and OB-cadherin (CDH11) are clinically correlated with breast cancer (18, 19). Aberrant methylation of H-cadherin (CDH13) associated with gene silencing has been reported in several primary tumors including breast and lung cancer (20). E-cadherin is intimately related to malignancy which is characterized by uncontrolled proliferation, dedifferentiation, invasion and metastasis, and its expression is reduced by hypermethylation of the promoter region in a number of tumors (17, 21). Furthermore, a cluster of cadherin genes are located on chromosome 16q, which is commonly deleted in various types of human cancer including HCC, with deletion rates ranging from 28% to 70% (22, 23). Therefore, it has been proposed that genetic and epigenetic alterations in CDH might be involved in liver carcinogenesis (24-26).

In this study, we examined the methylation status of CDH genes, located at 16q, using a methylation-specific polymerase

chain reaction (PCR), and evaluated the correlation with clinicopathological parameters.

Materials and Methods

Patients and specimens. Cancerous tissues and the surrounding non-cancerous hepatic parenchyma were obtained from 47 primary HCC patients who underwent resection at Nagoya University Hospital, Japan, during the period from May 1994 to December 1997. The study was approved by the Ethics Committee of the hospital. Informed consent was obtained from all patients for the subsequent use of their resected tissues. There were 43 male and 4 female patients, with ages ranging from 21 to 77 years (mean, 61 years). The mean follow-up period in the prognosis study was 64.3±31.9 (mean±SD) months. Tumor sizes ranged from 1.2 cm to 14.0 cm, with a mean size of 3.9±2.7 cm. Specimens were classified histologically using the Japanese staging system of the Liver Cancer Study Group of Japan (27).

Bisulfite modification and methylation-specific polymerase chain reaction (MSP). DNA from primary HCC tissues and corresponding non-cancerous tissues were subjected to bisulfite treatment. Briefly, 2 μg of DNA were denatured with NaOH and modified with sodium bisulfite. DNA samples were then purified using the Wizard purification resin (Promega Corp., Madison, WI, USA), treated again with NaOH, precipitated with ethanol, and resuspended in water. The modified DNA was used as a template for MSP. The PCR amplification of modified DNA samples consisted of 35 cycles of 94˚C for 15 s, 60˚C for 12 s and 72˚C for 10 s, after the initial denaturation step (94˚C for 5 min).

RT-PCR. The expression of M-cadherin mRNA was analyzed using RT-PCR. Total RNA (10 μg) isolated from primary HCC tissues and corresponding non-cancerous tissues were used to generate complementary DNA (cDNA) and then amplified by PCR primers for M-cadherin: 5'-TGACTACGAGGGTGACGGCT-3'(forward) and 5'-GGAGTGGCATGTCCAGTTGC-3'(reverse), which amplify a 263 bp product. The PCR amplification consisted of 30 cycles of 94˚C for 15 s, 60˚C for 15 s and 72˚C for 12 s, after the initial denaturation step (94˚C for 5 min).

Statistical analysis. To analyze the correlation between M-cadherin methylation status and clinicopathological parameters, differences in the numerical data between the two groups were evaluated using Fisher's exact test or χ^2 test. Overall survival rates were calculated using the Kaplan-Meier method, and the difference in survival curves was analyzed using the log-rank test. Independent prognostic factors were analyzed with the Cox proportional hazards regression model in a stepwise manner. Data are expressed as mean±SD. The presence of a statistically significant difference was denoted by $p<0.05$.

Results

Hypermethylation of the CDH promoter region in HCC. To investigate hypermethylation of the CDH promoter region, MSP was performed in 47 primary HCC tissues and corresponding non-cancerous tissues. We examined 7 CDH genes on chromosome 16q. In primary tissues, hyper-methylation was detected at a ratio ranging from 0% to 55.3% (Figure 1A). In particular, M-cadherin (CDH15) was the most hypermethylated of all 7 CDH genes. Representative cases of HCC tissues are shown in Figure 1B.

Table I. *Methylation stutus and mRNA expression of M-cadherin in HCC.*

	M-cadherin			M-cadherin	
Case	Methylation status	RT-PCR	Case	Methylation status	RT-PCR
1	-	↓	25	-	↓
2	+	↓	26	+	↑
3	+	↓	27	-	↑
4	+	↑	28	+	→
5	-	↑	29	+	↓
6	-	↑	30	+	↑
7	+	→	31	-	↓
8	-	↓	32	+	→
9	+	→	33	-	↓
10	-	↓	34	-	↑
11	+	↓	35	+	↑
12	+	↓	36	+	↑
13	+	↑	37	-	↑
14	+	↑	38	-	↑
15	+	↓	39	-	↓
16	+	↓	40	+	↓
17	+	→	41	-	↑
18	-	→	42	+	↓
19	-	↓	43	-	↓
20	-	→	44	+	→
21	+	↓	45	-	↑
22	+	↓	46	+	→
23	+	→	47	-	→
24	-	↑			

Abbreviations: +: methylated; -: unmethylated; ↑: higher than normal liver; →: same as normal liver; ↓: lower than normal liver.

Expression of M-cadherin mRNA in HCC. We examined the expression of M-cadherin mRNA using RT-PCR in primary HCC tissues and corresponding non-cancerous tissues. In 20 (42.5%) of 47 cases, the M-cadherin expression of primary HCC tissues was less than that of corresponding non-cancerous tissues (Table I). Representative cases are shown in Figure 1C. In 20 cases with lower expression of M-cadherin mRNA, 11 cases (55%) were hypermethylated (Table I).

Correlation between M-cadherin methylation status and clinicopathological parameters in HCC. The association between M-cadherin methylation status and clinicopathological parameters in patients with HCC was analyzed using Fisher's exact test or χ^2 test statistically (Table II). M-cadherin methylation was seen in well-differentiated cancers, and more frequently in larger

A

Cadherins	CDH 1	CDH 3	CDH 5	CDH 8	CDH 11	CDH 13	CDH 15
Methylated cases (%)	6 (12.8%)	19 (40.4%)	0 (0%)	3 (6.4%)	5 (10.6%)	8 (17.0%)	26 (55.3%)

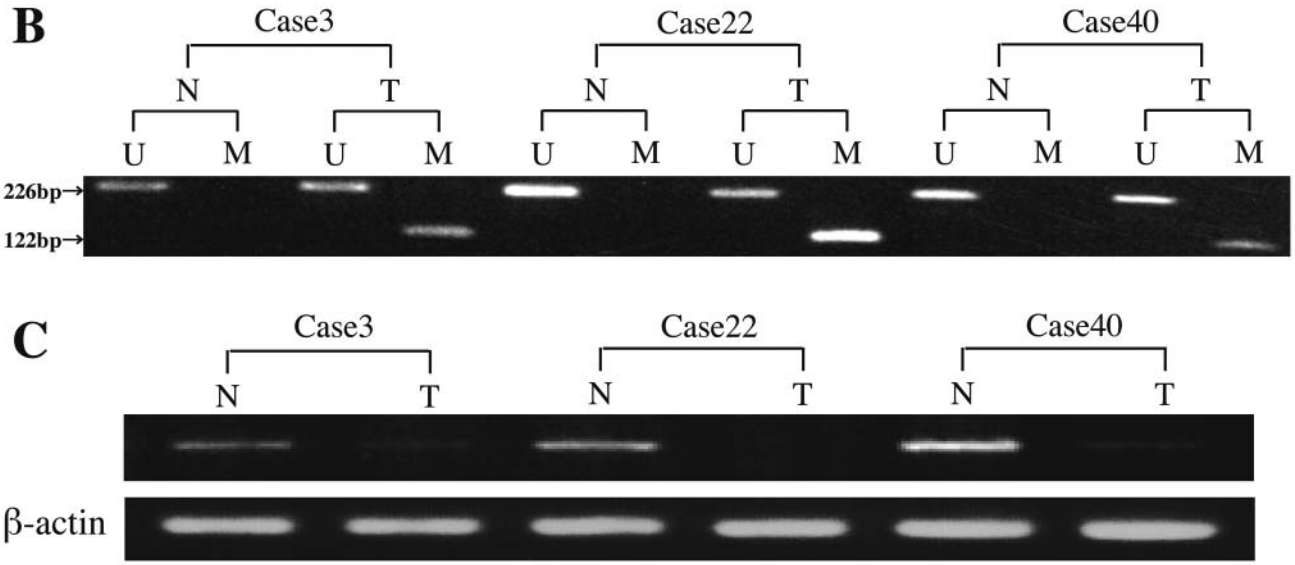

Figure 1. *A) Hypermethylation of CDH genes, located on 16q, in HCC. N: non-cancerous tissue, T: HCC tissue, U: Unmethylated, M: Methylated. B) Promoter hypermethylation of M-cadherin in HCC tissues. The representative cases are shown. C) The expression of M-cadherin mRNA in HCC tissues using RT-PCR.*

tumors, although the differences were not statistically significant. No significant association was found between M-cadherin methylation status and other variables.

Univariate and multivariate analyses of prognostic factors for patients with HCC. We analyzed the overall survival rates to assess the prognostic significance of M-cadherin methylation status. The five-year overall survival rate of the 47 patients with HCC was 74.1%. Patients with methylated M-cadherin had shorter 5-year survival rates than patients with unmethylated M-cadherin (overall survival rate, 67.4% *vs.* 82.7%; $p=0.0167$; Figure 2) when assessed using Kaplan-Meier curves.

To evaluate the potential role of M-cadherin methylation status in determining the postoperative prognosis of HCC patients, univariate analysis was conducted. The results showed that M-cadherin methylation was a significant predictor of overall survival ($p=0.0215$). Moreover, multivariate analysis was performed with 6 other prime variables (α-fetoprotein, tumor multiplicity, tumor size, vascular invasion, liver cirrhosis and pathological TNM stage), and it was revealed that M-cadherin methylation status and liver cirrhosis remained the strongest variables for independently predicting overall survival ($p=0.011$ and $p=0.024$, respectively; Table III).

Discussion

Cadherins form a cluster in this chromosomal region and are also considered prime candidates for tumor suppressor genes. M-cadherin, a calcium-dependent intercellular adhesion molecule, is expressed in skeletal muscle cells. It is important for skeletal muscle development, in particular the fusion of myoblasts into myotubes (28). E-cadherin mediates cell-cell interaction in a calcium-dependent manner. The extracellular domain of E-cadherin is important for cell-cell interaction, and the intracellular domain binds to catenins that attach to the actin cytoskeleton (9). In tumor model systems, down-regulation of E-cadherin has been associated with loss of intercellular

Table II. *The correlation between M-cadherin methylation status and clinicopathalogical parameters in HCC.*

Clinicopathological parameters	Number of cases	M-cadherin methylation (number)		*P*-value
		Methylated	Un-methylated	
Age				
<60	13	6	7	0.435
≥60	34	20	14	
Gender				
Male	43	25	18	0.202
Female	4	1	3	
Virus				
HBV	6	2	4	0.478
HCV	37	22	15	
None	4	2	2	
Pugh-Child's classification				
A	43	23	20	0.408
B	4	3	1	
AFP				
<20 ng/ml	17	9	8	0.883
≥20 ng/ml	29	16	13	
PIVKA II				
<0.1 AU/ml	23	13	10	0.847
≥0.1 AU/ml	15	8	7	
Tumor multiplicity				
Solitary	32	17	15	0.659
Multiple	15	9	6	
Histologic type of tumor				
Well-differentiated	5	5	0	0.062
Moderately-differentiated	41	20	21	
Poorly-differentiated	1	0	1	
Tumor size				
<3.5cm	29	13	16	0.063
≥3.5cm	18	13	5	
Pattern of tumor growth				
Expansive	40	22	18	0.832
Infiltrative	5	3	2	
Formation of fibrous capsule				
Present	38	21	17	0.707
Absent	8	5	3	
Capsular infiltration				
Present	36	20	16	>0.999
Absent	12	5	7	
Septal formation				
Present	37	19	18	0.222
Absent	8	6	2	
Vascular invasion				
Present	10	6	4	0.737
Absent	37	20	17	
Liver cirrhosis				
Present	17	8	9	0.391
Absent	30	18	12	
Surgical margin				
Negative	24	15	9	0.316
Positive	21	10	11	
Pathological Stage				
I	5	3	2	0.989
II	28	15	13	
III	9	5	4	
IVA	5	3	2	

HBV: hepatitis B virus; HCV: hepatitis C virus; AFP: alpha-fetoprotein; PIVKAII: protein induced by vitamin K absence or antagonists-II.

Table III. *Multivariate analysis for overall survival of patients with HCC.*

Variable	Overall survival		
	Odds ratio	95% Confidence interval	*P*-value
M-cadherin methylation status (present: absent)	4.47	1.40-14.2	0.011
AFP (≥20 ng/ml: <20 ng/ml)	1.45	0.56-3.76	0.447
Tumor multiplicity (multiple: solitary)	0.99	0.27-3.58	0.985
Tumor size (≥3.5 cm: <3.5 cm)	0.47	0.11-1.94	0.298
Vascular invasion (present: absent)	2.24	0.37-13.7	0.382
Liver cirrhosis (present: absent)	3.28	1.17-9.18	0.024
Pathological stage (I and II: III and IV)	1.45	0.17-12.5	0.736

adhesiveness, which may result in initiation of invasion, tumor dedifferentiation and the destruction of normal tissue morphology (17, 21). Other cadherin family genes are localized in this region and could also be involved in the tumorigenesis of liver cells. The present study provides the first evidence of reduced M-cadherin expression in HCC tissues, with the reduction corresponding to the reduced survival of HCC patients.

Because hypermethylation of the E-cadherin promoter has been observed at significant rates in HCC (25, 26), we examined the methylation status of CDH genes on chromosome 16q using MSP. In primary tissues, M-cadherin was the most hypermethylated of 7 CDH genes. Clinically, those patients with methylated M-cadherin displayed a significantly poorer prognosis. Because M-cadherin is mainly expressed in skeletal muscle cells and there was no association between methylation status and clinico-pathological parameters, it is difficult to explain this result. However, the cadherins form a cluster in this chromosomal region, in particular, M-cadherin is found near E-cadherin, and we suspect that this fact could explain the result. Further study of the mechanisms between cadherins and functional research of M-cadherin is needed.

Conclusion

M-cadherin is hypermethylated in HCC and is significantly associated with overall survival. Detection of M-cadherin hypermethylation in surgically excised HCC tissues may provide a new strategy for cancer prevention, an adjuvant therapy, and a potential biomarker for the prognostication of HCC. Further investigation is needed to understand how M-cadherin methylation contributes to the pathogenesis of HCC.

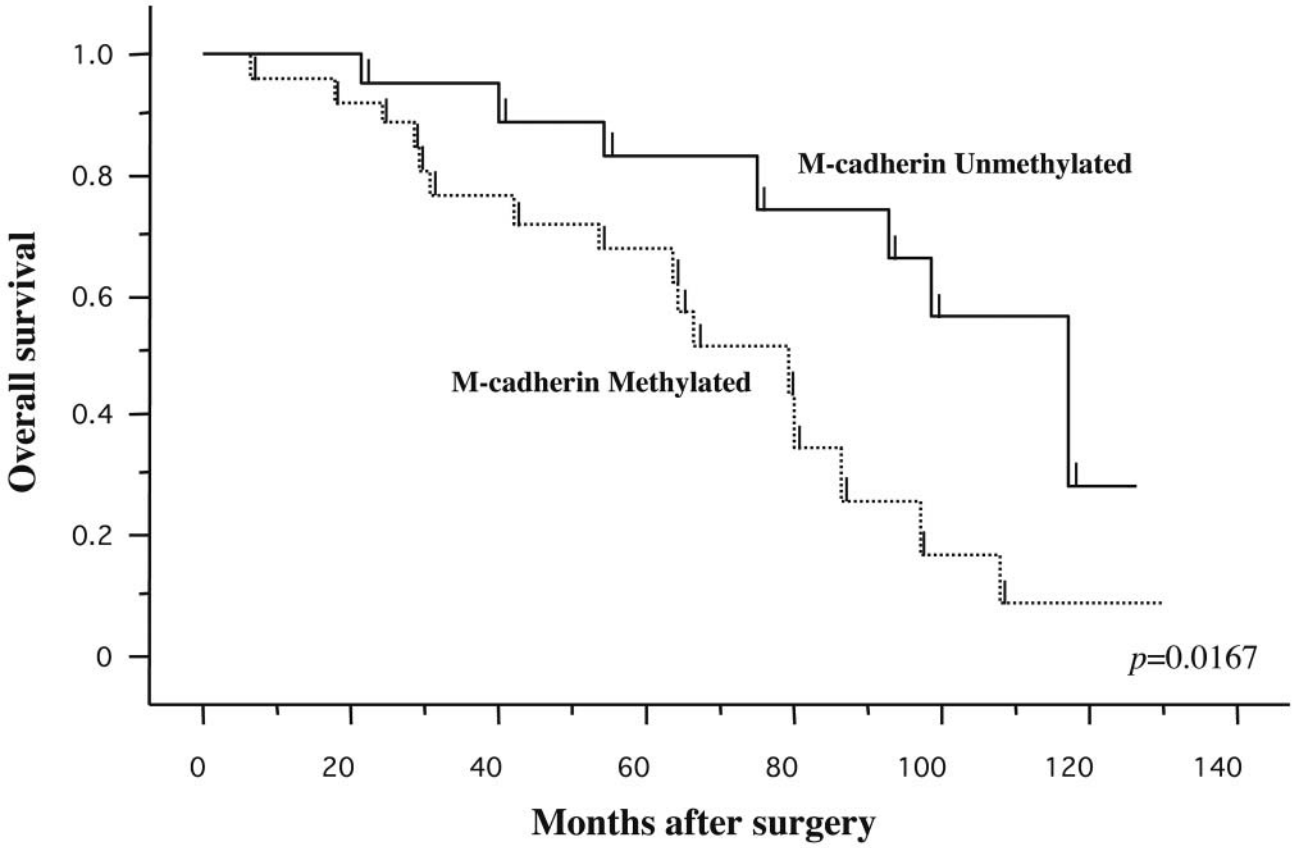

Figure 2. *Patients with methylated M-cadherin had shorter 5-year survival rates than patients with unmethylated M-cadherin (overall survival rate, 67.4% vs. 82.7%; p=0.0167) when assessed using Kaplan-Meier curves.*

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