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Expression of DNA Methyltransferase (DNMT) 1, 3a and 3b Proteins in Human Hepatocellular Carcinoma

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Alteration of aberrant DNA methylation is one of the most consistent epigenetic changes found in human cancers. DNA methylation is catalyzed by DNA methyltransferase (DNMT). In this study, we examined DNMT protein expression by immunohistochemistry in surgically resected hepatocellular carcinomas (HCCs). Sections of paraffin-embedded specimens were obtained from 95 patients with HCC between 1989 and 2002. The specimens were stained with anti-DNMTs (DNMT1, DNMT3a and DNMT3b) antibodies. There were statistically significant associations between DNMT protein expression and tumor differentiation ($P < 0.05$) and intrahepatic metastasis ($P < 0.05$). DNMT3a protein expression was significantly correlated with portal vein involvement of tumors ($P < 0.05$). The overall survival rates of patients with DNMT3a-positive HCCs and DNMT3b-positive HCCs were significantly lower than those of patients negative for these proteins ($P < 0.005$, respectively). To further evaluate the correlation between DNMT protein expression and patient survival, we classified patients into 3 groups: Group 1, DNMT1(+), 3a(–) and 3b(–); Group 2) DNMT1(+), 3a or 3b(+); and Group 3) DNMT1(+), 3a(+) and 3b(+). The overall survival rate of patients in Group 3 was significantly lower than those of patients in Groups 1 and 2 ($P = 0.0009$). In conclusion, the results of this study suggest that DNMT1, DNMT3a and DNMT3b are cooperatively involved in determining the extent of HCCs, and that DNMT protein overexpression in HCCs may be a predictive factor for poor survival.

Key words: DNA methyltransferase; hepatocellular carcinoma; immunohistochemistry; prognosis

DNA methylation plays an important role in the regulation of gene expression, and gene silencing by aberrant hypermethylation of CpG islands in gene promoter regions frequently occurs in human cancers. DNA methyltransferases are involved in the transfer of methyl groups to promoter CpG islands, and many tumor suppressor genes can be silenced by promoter CpG island methylation. Alteration of aberrant DNA methylation is one of the most consistent epigenetic changes found in human cancers (Girault et al., 2003; Ding et al., 2008; Nosho et al., 2009).

DNA methyltransferases have been reported to be encoded by 3 distinct families of DNMT genes: DNMT1, DNMT2, and DNMT3. DNMT1 is the best known DNMT involved in the maintenance of methylation, while DNMT3 is involved in de novo methylation and is encoded by 2 distinct genes, DNMT3a and DNMT3b (Łuczak and Jagodzinski, 2006). The function of DNMT2

Abbreviations: CH, chronic hepatitis; DNMT, DNA methyltransferase; HCC, hepatocellular carcinoma; LC, liver cirrhosis; siRNA, small interfering RNA

Table 1. Relationship between DNMTs and tissue specimens

Patients	*n*	Immunostaining score					
		DNMT1		DNMT3a		DNMT3b	
Age (year)							
< 60	39	2.179 ± 0.721	NS	1.897 ± 0.882	NS	1.205 ± 0.656	NS
≥ 60	56	2.357 ± 0.586		1.786 ± 0.847		1.232 ± 0.831	
Sex							
Male	76	2.263 ± 0.640	NS	1.776 ± 0.873	NS	1.145 ± 0.905	NS
Female	19	2.368 ± 0.684		2.053 ± 0.800		1.526 ± 0.905	
Background of cancerous liver tissue							
Chronic hepatitis	27	2.370 ± 0.655	NS	1.556 ± 1.013	NS	1.000 ± 0.679	NS
Liver cirrhosis	68	2.250 ± 0.629		1.941 ± 0.770		1.309 ± 0.778	
Tumor differentiation							
Well differentiated	19	1.842 ± 0.765	$P < 0.05$	1.421 ± 0.769	$P < 0.05$	0.895 ± 0.657	$P < 0.05$
Moderately differentiated	59	2.339 ± 0.633		1.814 ± 0.880		1.254 ± 0.779	
Poorly differentiated	17	2.471 ± 0.624		2.235 ± 0.831		1.412 ± 0.870	
Portal vein involvement of tumor							
Positive	51	2.314 ± 0.678	NS	2.000 ± 0.849	$P < 0.05$	1.294 ± 0.782	NS
Negative	44	2.250 ± 0.615		1.636 ± 0.838		1.136 ± 0.734	
Pathological findings Fc-inf							
Positive	68	2.319 ± 0.675	NS	1.870 ± 0.873	NS	1.188 ± 0.791	NS
Negative	27	2.192 ± 0.567		1.731 ± 0.827		1.308 ± 0.679	
Intrahepatic metastasis							
Positive	28	2.571 ± 0.504	$P < 0.05$	2.143 ± 0.803	$P < 0.05$	1.464 ± 0.693	$P < 0.05$
Negative	67	2.164 ± 0.665		1.701 ± 0.853		1.119 ± 0.769	
Tumor size							
≤ 20 mm	22	2.136 ± 0.640	NS	1.864 ± 0.774	NS	1.273 ± 0.767	NS
> 20 mm or < 50 mm	50	2.400 ± 0.670		1.800 ± 0.948		1.180 ± 0.800	
≥ 50 mm	23	2.087 ± 0.733		1.783 ± 0.723		1.217 ± 0.736	

n, number of patients.
Fc-inf, infiltration to the capsule; NS, not significant.

remains to be determined. Interactions among DNMTs and methyl CpG-binding domain proteins should also be considered as a mechanism for maintaining DNA methylation patterns using the proofreading or remethylation function (Hattori et al., 2004).

Aberrant hypermethylation of CpG islands has recently been implicated in hepatocarcinogenesis, and it has been reported that tumor suppressor genes in hepatocellular carcinomas (HCCs) are affected by such silencing (Saito et al., 2001; Yang et al., 2003). To study the significance of aberrant DNMT (DNMT1, 3a and 3b) expression in human HCC, we evaluated the correlation between DNMT protein expression and clinicopathologic features and prognosis in patients with HCC.

Materials and Methods

Patients and samples

Stored HCC and corresponding non-cancerous tissue specimens from our laboratory were obtained for 95 patients from 1989 to 2002. The protocol was approved by the ethics committee of the School of Medicine, Tottori University (1040). Informed consent was obtained from all patients.

The clinicopathologic features of the 95 patients are shown in Table 1. A mean age was 61 ± 10 years (range, 17–80 years). For comparison, normal liver tissues were also obtained from 3 patients who had undergone surgical resection for hepaticolithiasis.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut into 4-µm-thick continuous sections and mounted on poly-L-lysine-coated glass slides. Slides were deparaffinized in xylene, rehydrated in graded alcohols and washed in tap water. Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2. Slides were heated in a microwave at 600 W for 15 min with 10 mM sodium citrate buffer (pH 6.0) and treated with polyoxyethylene sorbitan monolaurate (Tween 20) for 30 min for antigen retrieval. Rabbit polyclonal antibodies for DNMT1 (H-300, sc-20701, dilution 1:200), DNMT3a (H-295, sc-20703, dilution 1:200) and DNMT3b (H-230, sc-20704, dilution 1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) were applied overnight, followed by incubation with biotinylated secondary antibodies [anti-rabbit IgG, N-Histofine, Simple Stain MAX PO (MULTI)] at room temperature for 30 min. Antigen-antibody complexes were visualized using a streptavidin-horseradish peroxidase conjugate (LSAB kit; Dako, Carpinteria, CA) with diaminobenzidine as a chromogen. Slides were counterstained with Harris' hematoxylin and methylgreen for 3 to 5 s.

Scoring of immunoreactivity

Two independent observers (T.M. and K.E.) independently evaluated the immunostaining without knowledge of the clinical outcomes; the concordance ratio was > 90%. When differences in scoring were encountered, a consensus of two was used to obtain a final decision.

Nuclear positivities for the 3 proteins were evaluated using the scoring system previously reported by Choi et al. (2003), according to staining intensity (0, negative; 1, weak; 2, moderate; and 3, severe) and proportion of positive cells (0, negative; 1, positive in ≤ 10%; 2, positive in > 10% and ≤ 33%; 3, positive in > 33% and ≤ 66%; 4, positive in > 66% of cells). The 2 scores were added in each case, and the expression was graded as – (0), 1+ (1 or 2), 2+ (3 to 5) and 3+ (6 or 7).

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad Software, La Jolla, CA). Spearman's rank correlation test and Student's *t*-test were used to study the relationships between DNMT immunoreactivities and various clinicopathologic parameters. Overall survival rates were determined by the Kaplan-Meier method, and the significance of differences between survival rates was studied using the log-rank test. Differences or correlations with P values < 0.05 were considered significant. Positive DNMT expression was defined as moderate (2+) or strong (3+), and negative DNMT expression as absent (–) or weak (1+) expression. To further evaluate the correlation between DNMT protein expression and patient survival, we subdivided the patients into 3 groups: Group 1) DNMT1(+), 3a(–) and 3b(–); Group 2) DNMT1(+), 3a or 3b(+); and Group 3) DNMT1(+), 3a(+) and3b (+).

Results

The immunohistochemical staining results are shown in Table 2 and Fig. 1. The non-cancerous liver tissues showed no expression of nuclear DNMT proteins by immunohistochemistry. There were no differences in cytoplasmic DNMT expres-

Table 2. Expression of DNMT 1, 3a and 3b proteins in patients with hepatocellular carcinoma

	DNMT protein expression							
	–		1+		2+		3+	
	n	(%)	*n*	(%)	*n*	(%)	*n*	(%)
DNMT1	0	(0)	10	(10.5)	50	(52.6)	35	(36.8)
DNMT3a	8	(8.4)	20	(21.1)	48	(50.5)	19	(20.0)
DNMT3b	14	(14.7)	51	(53.7)	25	(26.3)	5	(5.2)

n, number of patients: total number, 95.
Nuclear positivities for the 3 proteins were evaluated using the scoring system, according to staining intensity (0, negative; 1, weak; 2, moderate; and 3, severe) and proportion of positive cells (0, negative; 1, positive in ≤ 10%; 2, positive in > 10% and ≤ 33%; 3, positive in > 33% and ≤ 66%; 4, positive in > 66% of cells). The 2 scores are added in each case, and the expression is graded as – (0), 1+ (1 or 2), 2+ (3 to 5) and 3+ (6 or 7).

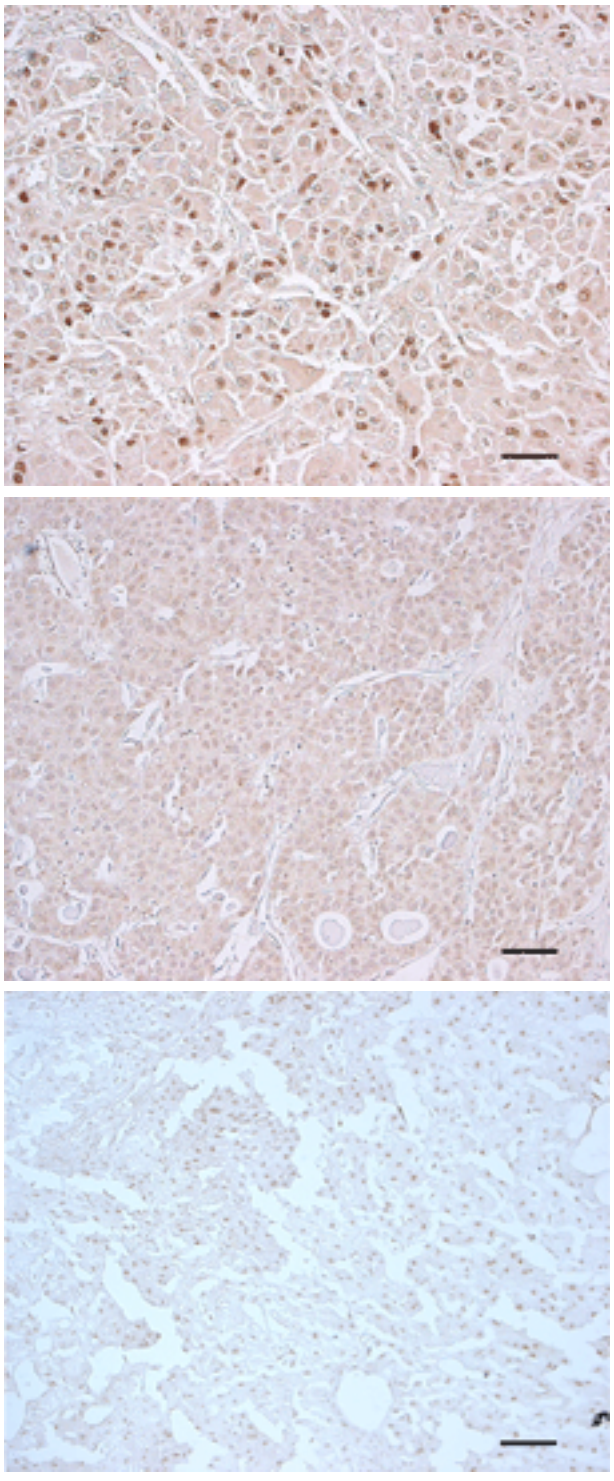

Fig. 1. Immunohistochemical staining results for (**a**) DNMT1, (**b**) DNMT3a and (**c**) DNMT3b proteins in hepatocellular carcinomas. Strong nuclear expression and faint cytoplasmic expression are seen in these 3 cases. **a** and **b:** counterstained with Harris' hematoxylin. **c:** conterstained with methylgreen. Bar = 100 µm.

sion between patients with CH and LC (data not shown). None of the 3 normal control livers demonstrated nuclear expression of DNMTs.

We indicated the immunostaining scores according to the clinicopathological findings in Table 1. There were significant associations between DNMT protein expression and tumor differentiation or intrahepatic metastasis. DNMT3a protein expression was significantly correlated with portal vein involvement in the tumor. On the other

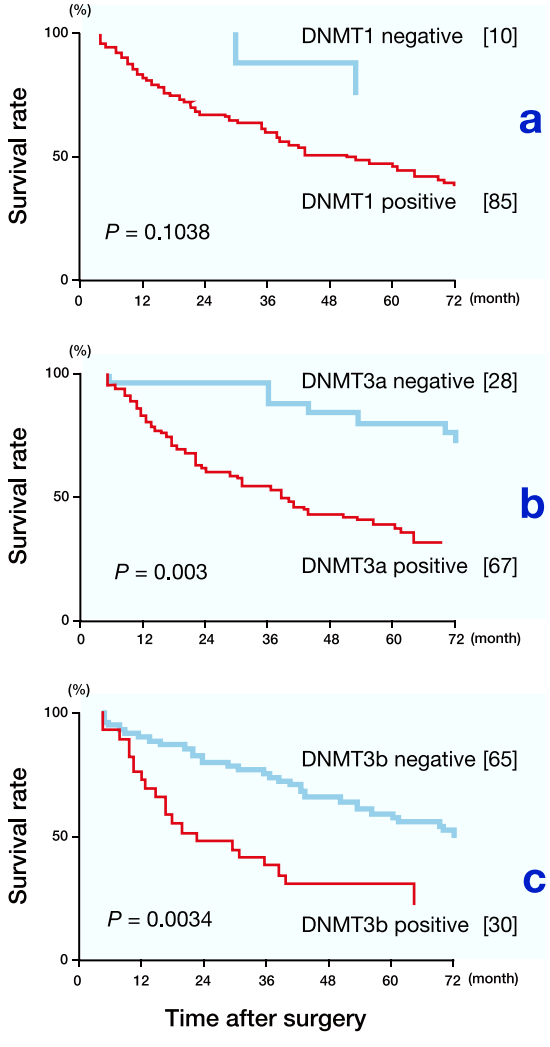

Fig. 2. Kaplan-Meier survival curves for patients with hepatocellular carcinomas according to DNMT protein expression levels. [ ], number of patients.

a: DNMT 1.
b: DNMT 3a.
c: DNMT 3b.

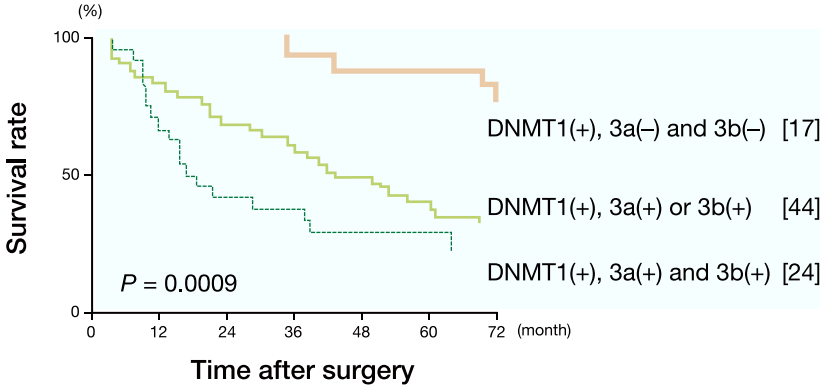

Fig. 3. Kaplan-Meier survival curves were plotted for 94 patients according to the correlation with expression of the DNMT protein complex. [ ], number of patients.

Group 1: DNMT1(+), 3a(–) and 3b(–).
Group 2: DNMT1(+), 3a or 3b(+).
Group 3: DNMT1(+), 3a(+) and 3b(+).

hand, no significant correlations were noted between DNMT1 or 3b and portal vein involvement. There were no significant associations between DNMT expression and age, sex, tumor size, infiltration to the capsule or underlying liver disease.

Follow-up information was available for all 95 patients, covering periods ranging from 0 to 183 months (mean, 63.7 months). Kaplan-Meier survival curves were plotted for the 95 patients according to expression of each DNMT.

The overall survival rates of DNMT3a-positive and DNMT3b-positive HCC patients were significantly lower than those of patients negative for these proteins (Fig. 2b, $P = 0.003$, log-rank test; Fig. 2c, $P = 0.0034$, log-rank test). However, the overall survival rate of DNMT1-positive HCC patients was not significantly different from that of DNMT1-negative HCC cases (Fig. 2a, $P = 0.1038$, log-rank test).

The overall survival rate of patients in Group 3 [DNMT1(+), 3a(+) and 3b(+)] was significantly lower than those of patients in Groups 1 and 2 (Fig. 3, $P = 0.0009$, log-rank test).

Discussion

DNA methylation is well known to be involved in the early developmental stages of HCC with a background of chronic liver disease, including CH and LC. It has recently been suggested that aberrant DNA methylation of CpG islands is an early and frequent event, and that stepwise progression of methylating events contributes to multistep hepatocarcinogenesis (Roncalli et al., 2002; Lee et al., 2003; Yang et al., 2003). The levels of DNMT1, DNMT3a and DNMT3b mRNA in HCCs are significantly higher than in noncancerous liver tissues, including normal, chronic CH and LC tissues (Saito et al., 2001; Oh et al., 2007).

In the present study, we used an immunohistochemical technique to directly examine the protein expression of 3 DNMTs in HCCs. The results demonstrated that DNMT protein expression in HCCs was significantly higher than in noncancerous liver tissues including normal, CH and LC tissues. In addition, there was no significant relationship between DNMT expression, CH and LC.

Immunohistochemical staining showed very strong nuclear expression and faint cytoplasmic expression of DNMT1, and strong nuclear and faint cytoplasmic expression of DNMT3a and DNMT3b. Some immunohistochemical staining of these DNMTs has previously been performed in a variety of cancers (Girault et al., 2003; Lin et al., 2007; Ding et al., 2008) and HCC (Saito et al., 2003; Oh et al., 2007). Although nuclear and cytoplasmic DNMT3b expression has been detected in other cancers, no results of immunohistochemical staining of DNMT3b have previously been reported in HCC.

The present study showed a significant relationship between DNMT3a protein expression and portal vein involvement, and significant rela-

tionships between DNMT protein expression and tumor differentiation or intrahepatic metastasis. However, a previous report found no significant relationship between DNMT mRNA levels and portal vein involvement and tumor differentiation in HCCs (Saito et al., 2001), but the same research group reported a significant relationship between DNMT1 protein expression and portal vein involvement or tumor differentiation (Saito et al., 2003). Reverse transcriptase-polymerase chain reaction is sufficiently sensitive to detect small elevations in DNMT mRNA levels, while immunohistochemistry is only able to detect elevations in protein expression when they reach a certain level in more malignant HCCs. We suggest that DNMT protein expression may be more strongly correlated with progression of the cancerous stage of HCCs, rather than the precancerous stage. To the best of our knowledge, there have been no reports of any significant relationship between DNMT protein expression and intrahepatic metastasis, and there have been some reports of a lack of any significant relationship between DNMT protein expression and lymphogenous metastasis in other cancers (Ding et al., 2008; Nosho et al., 2009). However, the relationship between DNMT protein expression and hematogenous metastasis has not been previously reported. E-cadherin, an intercellular adhesion-related protein, is coded for by a tumor suppressor gene. E-cadherin gene methylation has been associated with reduced expression of the protein, resulting in a loss of intercellular adhesion and tissue destruction in HCCs (Kanai et al., 1997). In a recent study, E-cadherin expression was reported to be significantly increased after concomitant siRNA-mediated depletion of DNMT1, 3a and 3b in endometrial epithelial carcinoma cells (Rahnama et al., 2009). DNMTs are thought to be involved in E-cadherin methylation. In addition, reduction of E-cadherin may also be related to the acquisition of cell motility, tumor cell invasion and vascular invasion. E-cadherin gene methylation in HCCs has been shown to be associated with a poor prognosis (Kozyraki et al., 1996; Lee et al., 2003). DNMT protein expression may therefore contribute to hematogenous metastasis in HCC.

We investigated the relationship between expression of the 3 DNMTs and overall patient survival. This is the 1st report to reveal poor overall survival in HCC patients with positive immunoreactivity for DNMT3a and DNMT3b protein expression. No significant relationship between DNMT1 protein expression and overall patient survival was noted, though some studies on HCC and lung cancers have shown a significant relationship between DNMT1 protein expression and overall survival (Saito et al., 2003; Lin et al., 2007). The reasons for this discrepancy remain to be determined. DNMT1 is referred to as a "maintenance" methyltransferase, and DNMT1 protein expression may thus not contribute to overall survival. However, our study demonstrated a significant relationship between protein expression of the 3 DNMTs and poor overall survival in HCCs. It was recently reported that DNMT1 and DNMT3a/3b may associate directly and function as a complex for DNA methylation of CpG islands (Kim et al., 2002; Rhee et al., 2002; Hattori et al., 2004). The results of the present study suggest that DNMT1, DNMT3a and DNMT3b may be cooperatively involved in determining the extent of HCCs. Thus each DNMT could affect survival in patients with HCC.

DNA methylation is known to be a reversible process. In a previous study, promoter methylation by DNMT was shown to be reversible using the DNMT inhibitor 5-aza-2'-deoxycytidine, which induced re-expression of promoter-methylated genes in a cell line in vitro (Juttermann et al., 1994; Yang et al., 2001; Arai et al., 2006). 5-Aza-2'-deoxycytidine and 5-azacytidine in non-toxic doses have been approved by the United States Food and Drug Administration as DNMT inhibitors for hematological malignancies (Mulero-Navarro and Esteller, 2008). The relationship between DNMT protein expression and patient prognosis demonstrated in the present study suggests a potentially important role for DNMT inhibitor therapy in HCC patients with an increased DNMT protein expression.

In conclusion, the present study suggests that overexpression of DNMT proteins in HCCs may be a predictive factor for poor survival. In addition, DNMTs may represent new prognostic markers and potential therapeutic targets in HCC.

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