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Increased DNA Methyltransferase Expression Is Associated with an Early Stage of Human Hepatocarcinogenesis

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The present study was designed to determine whether changes in DNA methyltransferase (DNA MTase) expression are involved in hepatocarcinogenesis. We examined DNA MTase expression in normal liver tissue (with no remarkable histological findings), liver tissue showing chronic hepatitis or cirrhosis, which are generally thought to be precancerous conditions, and hepatocellular carcinomas (HCCs) using the reverse-transcriptase polymerase chain reaction assay. DNA MTase mRNA levels were significantly higher in liver tissue showing chronic hepatitis and cirrhosis (DNA MTase mRNA/ β-actin mRNA ratio $=0.30\pm0.22$, $n=24$, $P<0.01$) than in normal liver tissue either from patients with liver metastatic lesions of colonic cancer (0.14 ± 0.05, $n=6$) or from patients with HCCs (0.16 ± 0.07, $n=3$). DNA MTase mRNA levels were even higher in HCC tissue (0.34 ± 0.18, $n=29$). These results suggest that increased DNA MTase expression may be an early event during hepatocarcinogenesis. DNA MTase is a potential target for HCC preventive therapy.

Key words: DNA methyltransferase — Hepatocellular carcinoma — Chronic hepatitis — Cirrhosis

The majority of HCCs arise in chronically diseased livers, including livers with chronic hepatitis and cirrhosis resulting from hepatitis B or C virus infection or exposure to other carcinogenic factors. These chronic liver diseases are widely considered to be precancerous conditions.[1,2] The processes of chronic hepatitis and cirrhosis are characterized by features such as cell death, inflammation, regeneration and fibrosis. In these precancerous conditions, it is considered that certain molecular alterations occur, predisposing hepatocytes to malignant transformation. An understanding of the underlying mechanism of malignant transformation is essential for the development of preventive procedures that can slow or arrest the transformation from a precancerous condition to an HCC. However, little is known about this mechanism at present.

Changes in DNA methylation patterns, including global genomic hypomethylation and regional hypermethylation, occur consistently in human cancers.[3] Recently, we found that DNA hypermethylation on chromosome 16 occurs frequently not only in HCCs, but also in non-cancerous liver tissue showing chronic hepatitis and cirrhosis, suggesting that aberrant DNA methylation participates in an early stage of hepatocarcinogenesis.[2] The DNA methylation pattern is maintained by DNA MTase.[4,5] Cancer cell lines and human tumors express elevated levels of DNA MTase.[4,6,7] Regional DNA hypermethylation in cancer cells may result from increased activity of DNA MTase. To evaluate the possible role of DNA MTase in HCC development and progression, we examined DNA MTase expression in normal liver, chronically diseased liver and HCC tissue.

Abbreviations: HCC, hepatocellular carcinoma; MTase, methyltransferase; RT-PCR, reverse-transcriptase polymerase chain reaction.

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MATERIALS AND METHODS

Samples Twenty-nine HCCs and corresponding non-tumorous liver tissue samples were obtained from 27 patients who underwent surgery at the National Cancer Center Hospital, Tokyo. The 29 tumors were classified into 8 early HCCs, representing the *in situ* or microinvasive stage of cancer,[8–10] and 21 advanced HCCs including 3 well, 8 moderately and 10 poorly differentiated tumors. Histological examination of the non-tumorous liver tissue from HCC patients revealed no remarkable findings in 3 samples and findings compatible with chronic hepatitis and cirrhosis in 11 and 8 tissue samples respectively. Six normal liver tissue samples were also obtained from patients who underwent partial hepatectomy for liver metastatic lesions of colonic cancer. Eight HCC cell lines, HepG2, PLC/PRF/5, Li7NM, Li7HM, Li21, Li22, Li23 and Li24,[11–13] were also used.

RT-PCR assay DNA MTase expression in HCCs and non-tumorous liver tissue was evaluated using a semi-quantitative RT-PCR assay as described previously.[14] Briefly, cDNA was synthesized from 100 ng of total RNA using random hexadeoxynucleotide primers (Takara Shuzo, Otsu) and was amplified using AmpliTaq DNA polymerase (Perkin Elmer, Branchburg, NJ). Primers used to detect human DNA MTase gene tran-

scripts were 5′-ATCTAGCTGCCAAACGGAGG-3′ (sense) and 5′-CACTGAATGCACTTGGGAGG-3′ (antisense), which yielded a 192-bp product. These primers were designed based on the human DNA MTase gene sequence.[5] PCR was performed for 30 cycles using cycle stages of 94°C for 30 s, 56°C for 60 s and 72°C for 90 s. To normalize the measurements of the amount and quality of the RNA, β-actin was also amplified from each sample using the primers 5′-CGAGCGGGAAATCGTGCGTGACATTAAGGAGA-3′ (sense) and 5′-TACCACTGGCATCGTGATGGACTGCGGTGACG-3′ (antisense) and the same PCR conditions, but for 27 cycles. Under these conditions, the yield of PCR products was proportional to the starting amount of template RNA. As a negative control, omission of reverse transcriptase resulted in no subsequent amplification signal. The reaction products were separated electrophoretically on a 3% agarose gel, and stained with ethidium bromide. Evaluation of the signal intensities was performed on a FMBIO-100 image analyzer (Takara Shuzo). The level of DNA MTase mRNA was expressed as the ratio of DNA MTase/β-actin mRNA.

Statistical analysis Data are presented as the mean±SD and were analyzed statistically using Student's t test. P values less than 0.05 were considered significant.

RESULTS

The average DNA MTase/β-actin mRNA ratio for normal liver tissue from 6 patients with liver metastatic lesions of colonic cancer was 0.14±0.05 (0.10, 0.12, 0.11, 0.11, 0.18, and 0.24; Figs. 1 and 2) and a similar value, 0.16±0.07 was obtained for 3 non-tumorous liver tissue samples without remarkable histological findings from HCC patients (patients 6, 20 and 21, Table I and Fig. 2).

Non-tumorous liver tissue showing chronic hepatitis or cirrhosis had significantly higher DNA MTase mRNA levels (0.30±0.22, $n=24$, $P<0.01$) than normal liver tissue from patients with liver metastatic lesions of colonic cancer or HCCs (Figs. 1 and 2). The DNA MTase mRNA levels of non-tumorous liver tissue showing chronic hepatitis or cirrhosis were not significantly different from each other (Table II). The DNA MTase mRNA levels were not significantly associated with the hepatitis virus type (Table I).

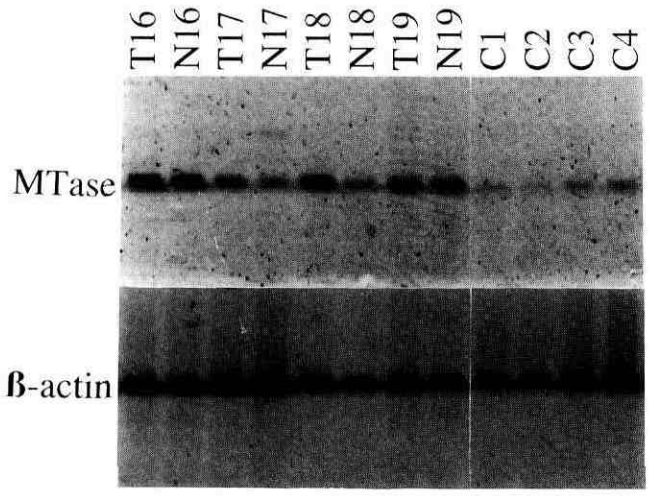

Fig. 1. RT-PCR products for DNA MTase and β-actin in tumors (T) and non-tumorous liver tissue showing chronic hepatitis or cirrhosis (N) from HCC patients (patients 16–19) as well as in normal liver tissue from patients with liver metastatic lesions of colonic cancer (C). cDNAs transcribed from 100 ng of total RNA were amplified using primers specific for DNA MTase and β-actin sequences. RT-PCR products were separated electrophoretically on an agarose gel, and stained with ethidium bromide, then the signal intensities were measured using an image analyzer (FMBIO 100). Higher DNA MTase mRNA levels were observed in HCCs and chronically diseased liver tissue than in normal liver tissue.

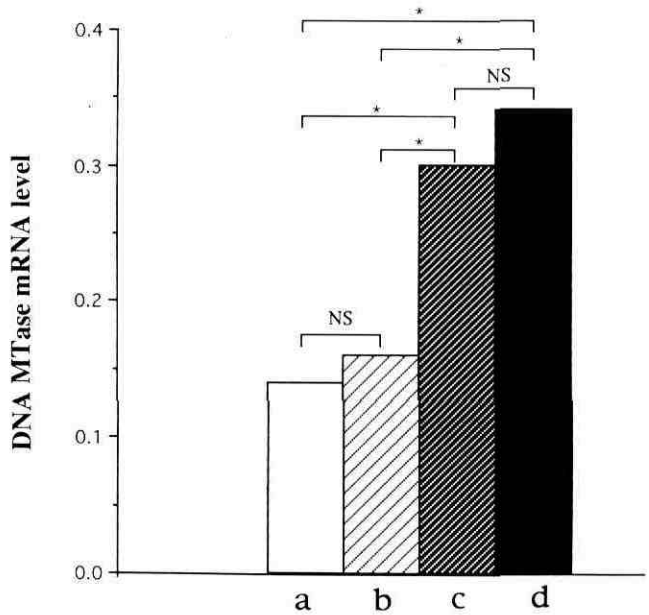

Fig. 2. DNA MTase mRNA levels. Lane a, normal liver tissue from patients with liver metastatic lesions of colonic cancer ($n=6$); b, normal liver tissue from patients with HCC ($n=3$); c, liver tissue showing chronic hepatitis or cirrhosis from patients with HCC ($n=24$); d, HCC tissue ($n=29$). * $P<0.01$. NS, not significant.

Table I. DNA Methyltransferase mRNA Expression in HCCs and Clinicopathological Parameters

Patients[a]	Hepatitis virus	Diagnosis of non-tumorous liver	Tumor differentiation	DNA MTase/β-actin ratio		
				Tumor	Non-tumor	T/N
1	C	CH	M	0.87	0.44	2.0
2	C	CH	P	0.20	0.17	1.2
3	B	CH	M	0.22	0.20	1.1
4	C	CH	P	0.20	0.19	1.1
5	C	LC	P	0.11	0.03	3.3
6	—	NL	P	0.31	0.12	2.6
7	C	CH	M	0.33	0.63	0.5
8	C	LC	W	0.18	0.11	1.6
9	C	CH	P	0.32	0.86	0.4
10	—	LC	P	0.18	0.13	1.5
11	C	CH	P	0.31	0.15	2.1
12	B and C	LC	W	0.33	0.09	3.8
13	C	CH	W	0.18	0.13	1.4
14	B	LC	M	0.20	0.16	1.2
15	B	CH	M	0.44	0.38	1.2
16	C	LC	M	0.81	0.78	1.0
17	—	CH	P	0.36	0.16	2.2
18	B	LC	P	0.49	0.44	1.1
19	C	LC	M	0.51	0.66	0.8
20	—	NL	M	0.16	0.13	1.2
21	B	NL	P	0.66	0.24	2.7
22	C	CH	W	0.30	0.24	1.3
23	C	LC	W	0.28	0.14	2.0
24 (T1)[b]	C	CH	W	0.42	0.26	1.6
(T2)			W	0.28		1.1
(T3)			W	0.34		1.3
25	C	LC	W	0.30	0.28	1.1
26	C	LC	W	0.30	0.25	1.2
27	C	LC	W	0.46	0.32	1.4
Average				0.34±0.18	0.28±0.22	1.6±0.8

a) Patients 1–21 had advanced HCCs and patients 22–27 had early HCCs.
b) Patient 24 had 3 HCC tumors: T1, T2 and T3.
Abbreviations: C, hepatitis C virus antibody positive; B, hepatitis B surface antigen positive; CH, chronic hepatitis; LC, liver cirrhosis; NL, normal liver; W, well differentiated HCC; M, moderately differentiated HCC; P, poorly differentiated HCC; T/N, ratio of tumor to corresponding non-cancerous liver tissue DNA MTase/β-actin mRNA ratios.

The DNA MTase mRNA levels were higher in HCCs (0.34±0.18, $n=29$) than in non-tumorous liver tissue showing chronic hepatitis or cirrhosis (Figs. 1 and 2). The DNA MTase mRNA levels were higher in 25 of the 29 HCCs than in non-tumorous tissue from the same patients; the increases were 2-fold or more in 8 of the HCCs (Table I, patients 1, 5, 6, 11, 12, 17, 21 and 23). Early HCCs (patients 22–27) showed DNA MTase mRNA levels (0.34±0.07) that were similar to the levels (0.35±0.21) in advanced HCCs (patients 1–21, Table I). DNA MTase expression did not significantly correlate with the degree of tumor differentiation (Table I).

Table II. DNA Methyltransferase mRNA Expression in Non-tumorous Livers

Diagnosis of non-tumorous liver	DNA MTase/β-actin ratio (mean±SD)
NL	0.16±0.07[a]
CH	0.33±0.22[b]
CL	0.28±0.23

a) $P<0.01$ compared with the values for CH and LC.
b) $P>0.1$ compared with the value for LC.
Abbreviations: NL, normal liver; CH, chronic hepatitis; LC, liver cirrhosis.

Additionally, all 8 HCC cell lines studied had DNA MTase mRNA levels (0.38 ± 0.06, range 0.29 to 0.46) that were similar to the levels observed in primary HCCs.

DISCUSSION

DNA MTase transfers a methyl group from *S*-adenosylmethionine to the 5-position of cytidine, and maintains the pattern of DNA methylation by recognizing and methylating hemimethylated DNA.[15] Many lines of evidence have indicated that an increase in DNA MTase expression is associated with cancer development and progression. First, DNA MTase gene expression is consistently higher in cultured cancer cells,[6] animal models of carcinogenesis[16, 17] and primary human cancers[4, 7] than in normal cells. Second, forced expression of exogenous DNA MTase cDNA in murine NIH 3T3 cells causes transformation,[18] and transfection of an antisense mRNA to the DNA MTase gene suppresses the tumorigenicity of the adrenocortical carcinoma cell line Y1.[19, 20] Third, a reduction in the DNA MTase activity in *Min* mice by biological and pharmacological methods dramatically reduces intestinal neoplasia formation.[21]

In this study, we focused on the involvement of DNA MTase in the development of human HCCs. We found that DNA MTase mRNA levels are significantly higher in livers showing chronic hepatitis or cirrhosis, which are generally thought to be precancerous conditions for HCCs, than in normal livers, and that DNA MTase levels were higher still in HCCs. These results suggest that increased DNA MTase expression may play a role in an early stage of hepatocarcinogenesis. To our knowledge, this is the first report of increased DNA MTase expression in precancerous conditions in humans. There was no significant difference in the DNA MTase mRNA levels between early and advanced HCCs, or between tumors with different degrees of differentiation, suggesting that increased DNA MTase expression may play a role in an early developmental stage rather than in the process of malignant tumor progression. In the present study, DNA MTase mRNA levels were high in livers showing chronic hepatitis or cirrhosis irrespective of the type of hepatitis virus infection. Increased DNA MTase expression has also been observed in livers from LEC rats with hepatitis. These rats are a mutant strain developed from the Long-Evans strain, and develop hepatitis due to abnormal copper metabolism, leading to spontaneous HCCs.[16] Taken together, these results suggest that increased DNA MTase expression may be a common event in an early stage of hepatocarcinogenesis independently of the kind of carcinogen.

The precise mechanisms by which increased DNA MTase expression contributes to the development of human cancers, either directly or through hypermethylation, remain to be determined, though there are at least 3 possibilities. First, regional DNA hypermethylation may cause chromatin configuration changes resulting in the loss of chromosome arms.[22–24] Second, DNA hypermethylation at specific loci in regulatory regions of genes leads to silencing of tumor suppressor genes and other genes related to cancer.[25–29] Third, C-to-T or corresponding G-to-A transition mutations at CpG sites may be facilitated by increased DNA MTase, since 5-methylcytosine is spontaneously deaminated to thymine, or DNA MTase may act directly as an endogenous mutagen.[30, 31] Findings compatible with these mechanisms have been reported for HCCs: regional DNA hypermethylation predisposes for a loss of heterozygosity on chromosome 16,[2] silencing of the E-cadherin gene is associated with CpG methylation around the promoter region,[32] and C-to-T and G-to-A transition mutations occur in the *p53* gene.[33, 34]

To prevent the development of HCCs in chronically diseased livers, biomedical scientists are focusing on the molecular events responsible for early developmental stages of HCCs, because early events are potential targets for biological preventive procedures. Although some molecular changes have been demonstrated in HCCs, only a few of them are thought to participate in the early stages of hepatocarcinogenesis.[2, 13] In the present study, we found that increased DNA MTase expression is an early event during HCC development. This finding is in agreement with our observation of regional DNA hypermethylation on chromosome 16 in livers showing chronic hepatitis and cirrhosis.[2] Changes in DNA methylation may be reversible. In experimental studies, reducing DNA MTase activity by biological and pharmacological measures has been shown to induce DNA demethylation and to inhibit tumorigenicity *in vitro* and *in vivo*.[19–21] Therefore, we propose DNA MTase as a candidate target for HCC preventive therapy.

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