Reduced mRNA Expression of the DNA Demethylase, MBD2, in Human Colorectal and Stomach Cancers

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A study was performed to evaluate the significance of aberrations of the newly identified DNA demethylase, MBD2, in human carcinogenesis. Levels of expression of DNA demethylase mRNA were examined by reverse transcription followed by real-time quantitative detection of the PCR products in 32 samples of colorectal cancer tissue, 24 stomach cancers, and the corresponding noncancerous mucosae. DNA demethylase mRNA levels normalized with glyceraldehydephosphate dehydrogenase (GAPDH) mRNA were reduced in 31 (97%) of the 32 colorectal cancers and in 22 (92%) of the 24 stomach cancers when compared with the levels in the corresponding noncancerous mucosae. The average levels of DNA demethylase mRNA expression normalized with GAPDH mRNA in each of the colorectal (0.81 ± 0.55) and stomach (2.88 ± 0.23) cancers were significantly lower than in the noncancerous mucosae (1.90 ± 0.16 and 5.11 ± 0.34, respectively, p < 0.0001). There was no significant association between the DNA demethylase mRNA level and malignant potential in both colorectal and stomach cancers. These data suggest that reduced expression of DNA demethylase may play a role at a certain step of multistage carcinogenesis. Reduction of DNA demethylase mRNA expression may be, if anything, one of the early events of carcinogenesis, but may not participate in the malignant progression of tumors.

Key Words: DNA methylation; DNA demethylase; MBD2; carcinogenesis.

Abbreviations used: RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehydephosphate dehydrogenase.

Moreover, an association between aberrant DNA methylation and chromosomal instability during carcinogenesis has been noted (8). Generally, the global DNA methylation level is lower in cancer cells than in normal cells (9) and some loci tend to show increased DNA methylation (10–16), whereas others are often hypomethylated in human cancers (17).

Until recently, a DNA methyltransferase, DNMT1, has been functionally defined as the only known enzyme determining DNA methylation levels in human cells (18). Preferences of DNMT1 for hemimethylated substrates compared to unmethylated substrates (19) and targeting of DNMT1 to replication foci (20) are believed to allow copying of the methylation pattern from the parental to the newly synthesized daughter DNA strand in somatic cells. mRNA expression and activity levels of DNMT1 are higher in precancerous lesions than in normal tissues, and they are further increased in cancerous tissues (21, 22). However, aberrations of DNA methylation patterns observed in cancers might not be solely attributable to increased levels of DNMT1.

Since DNMT1−/− embryonic stem cells still possess the ability to methylate viral DNA de novo (23), independently encoded de novo DNA methyltransferases have been sought. Recently, new DNA methyltransferases, DNMT2 (24), DNMT3a and DNMT3b (25), have been identified. In fact, the de novo DNA methylation activities of DNMT3a and DNMT3b have been confirmed, and the role of their overexpression in human carcinogenesis has been debated (26, 27).

Moreover, a DNA demethylase catalyzing the cleavage of a methyl residue from 5-methyl cytosine and its release as methanol has been newly identified, although reactions of this kind were originally believed to be cumbersome (28). The newly identified DNA demethylase is identical to MBD2, a recently described protein that contains a methyl CpG-binding domain homologous to the methyl CpG-binding domain of the MeCP2 transcriptional repressor (29). Although it has been proven that DNA demethylase shows CpG dinucleotide specificity, can demethylate CpG sites in dif-
fferent sequence contexts, and demethylates both fully methylated and hemimethylated DNA (30), detailed analyses of the function of DNA demethylase are still in their early stages. To our knowledge, aberrations of DNA demethylase in human cancers have not been described to date.

To reveal aberrations of DNA methylation patterns in human cancers, the expression of DNA demethylase mRNA in colorectal and stomach cancers and their corresponding non-cancerous mucosas was measured in this study using the quantitative reverse transcription-polymerase chain reaction (RT-PCR) method.

MATERIALS AND METHODS

Patients and tissue specimens. Paired specimens of primary colorectal cancer tissue and corresponding non-cancerous colorectal mucosa were obtained from surgically resected materials of 32 patients at the National Cancer Center Hospital, Tokyo, Japan. There were 21 men and 11 women with a mean age of 64 ± 11 (mean ± SD) years (range, 43 to 90 years). According to histological examination, 10 (31%), 18 (56%) and 4 (13%) colorectal cancers were classified as well, moderately and poorly differentiated adenocarcinomas, and 19 (59%) and 13 (41%) colorectal cancers were positive for vascular involvement and lymph node metastasis, respectively. Three (9%), 14 (44%), 12 (38%) and 3 (9%) cases were at Duke's A, B, C and D stages, respectively.

Paired specimens of primary stomach cancer tissue and corresponding non-cancerous stomach mucosa were obtained from surgically resected materials of 24 patients at the National Cancer Center Hospital, Tokyo, Japan. There were 21 men and 11 women with a mean age of 64 ± 11 (mean ± SD) years (range, 43 to 91 years). According to histological examination, 4 (17%), 8 (33%) and 12 (50%) stomach cancers were classified as well, moderately and poorly differentiated adenocarcinomas, and 24 (100%) and 23 (96%) stomach cancers were positive for vascular involvement and lymph node metastasis, respectively.

Quantitative RT-PCR analysis for DNA demethylase. The total RNAs were isolated from cancer tissue and corresponding non-cancerous mucosa using an acid guanidinium thiocyanate–phenol-chloroform method (33). First-strand cDNA was prepared from total cellular RNA using a random hexadexynucleotide primer and SuperScript RNase H− reverse transcriptase (GIBCO-BRL, Gaithersburg, MD). The following primer sets for the PCRs were designed with the support of Primer Express software (PE Biosystems, Foster City, CA). The primers used to amplify the DNA demethylase mRNA were 5'-AACCCCTGCTGTTTGGCTTAAC-3' (sense) and 5'-CGTACTGTCTGACCTGCTTC-3' (antisense), resulting in a 101-bp product. To confirm the quality of the RNA and to standardize the amount of RNA applied, glyceraldehydephosphate dehydrogenase (GAPDH) mRNA was amplified using 5'-GAAGATGGTGATGGGATTTC-3' (sense) and 5'-GAAGGTGAAGGTC-3' (antisense), resulting in a 226-bp product. For normalization with a gene associated with cell proliferation, histone H4 mRNA was also amplified using 5'-GCACTGAGGCTGACCAA-3' (sense) and 5'-GCCGAGTTCCACTCTTCCAAGA-3' (antisense), resulting in a 131-bp product. To prevent the reamplification of carryover PCR products, cDNAs were first treated with AmpErase uracil-N-glycosylase (PE Biosystems). The Hot Start technique was employed using AmpliTaq Gold DNA polymerase (PE Biosystems). The PCRs were performed using the SYBR Green PCR Core Reagents kit (PE Biosystems). Real-time detection of the emission intensity of SYBR Green bound into double-stranded DNA was performed by the GeneAmp 5700 Sequence Detection System (PE Biosystems). cDNAs from a stomach cancer cell line, MKN1 (34), were used as the calibrator samples. The relative quantification values were obtained from the threshold cycle number at which the increase in signal associated with an exponential growth of PCR products began to be detected using PE Biosystems analysis software, all according to the manufacturer's manuals. The quantitative PCRs were performed in triplicate for each sample-primer set, and the mean of the three experiments was used as the relative quantification value. At the end point of 40 PCR cycles, the reaction products were separated electrophoretically on a 3% agarose gel and stained with ethidium bromide. The signal intensity was not directly proportional to the amount of the template RNA in each sample, since this electrophoresis was performed at the end point of 40 PCR cycles.

RESULTS

Expression of DNA Demethylase mRNA in Colorectal Cancers

After the quantitative PCR using the GeneAmp 5700 Sequence Detection System, it was visually confirmed on agarose gels that specific products of about 101, 226 and 131-bp, and no nonspecific products, were obtained upon amplification of DNA demethylase, GAPDH and histone H4, respectively (Fig. 1). The signal intensity in Fig. 1 is not directly proportional to the amount of the template RNA in each sample, since this electrophoresis was performed at the end point of 40 PCR cycles.
cycles. The levels of DNA demethylase mRNA expression normalized with GAPDH mRNA in each sample from patients with colorectal cancer are shown in Fig. 2A. The levels were reduced in 31 (97%) of the 32 colorectal cancers when compared with the levels in the corresponding noncancerous mucosae. In 19 patients, DNA demethylase mRNA levels normalized with GAPDH mRNA were particularly low, being reduced by 50% or more when compared with the levels in the corresponding noncancerous mucosae. The average level of DNA demethylase mRNA expression normalized with GAPDH mRNA in colorectal cancers was significantly lower than that in the corresponding noncancerous mucosae (Table I, p < 0.0001). Levels of DNA demethylase mRNA normalized with histone H4 mRNA were reduced in 30 (94%) of the 32 colorectal cancers when compared with the levels in the corresponding noncancerous mucosae. In 23 patients, DNA demethylase mRNA levels normalized with histone H4 mRNA in colorectal cancers were reduced by 50% or more when compared with the levels in the corresponding noncancerous mucosae. The average level of DNA demethylase mRNA expression normalized with histone H4 mRNA in colorectal cancers was significantly lower than that in the corresponding noncancerous mucosae (Table I, p < 0.0001). Among colorectal cancers, there was no significant association between the DNA demethylase mRNA level and the clinicopathological parameters of the tumors, e.g., histological differentiation, presence or absence of vascular involvement, lymph node metastasis, and Dukes' classification.

Expression of DNA Demethylase mRNA in Stomach Cancers

As in colorectal cancers, specific PCR products were visually confirmed on agarose gels (Fig. 1). The levels of DNA demethylase mRNA expression normalized with GAPDH mRNA in each sample from patients with stomach cancer are shown in Fig. 2B. Levels of DNA demethylase mRNA normalized with GAPDH mRNA were reduced in 22 (92%) of the 24 stomach cancers

<table>
<thead>
<tr>
<th>Tissue samples</th>
<th>Normalized with glyceraldehydehyde 3-phosphate dehydrogenase</th>
<th>Normalized with histone H4</th>
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<tbody>
<tr>
<td>Colorectal cancers (n = 32)</td>
<td>1.90 ± 0.16</td>
<td>1.63 ± 0.26</td>
</tr>
<tr>
<td>Cancerous mucosae</td>
<td>0.81 ± 0.55</td>
<td>0.51 ± 0.07</td>
</tr>
<tr>
<td>Stomach cancers (n = 24)</td>
<td>5.11 ± 0.34</td>
<td>3.59 ± 0.527</td>
</tr>
<tr>
<td>Noncancerous mucosae</td>
<td>2.88 ± 0.23</td>
<td>1.16 ± 0.16</td>
</tr>
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* p < 0.0001.
when compared with the levels in the corresponding noncancerous mucosae. In 11 patients, DNA demethylase mRNA levels normalized with GAPDH mRNA in stomach cancers were particularly low, being reduced by 50% or more when compared with the levels in the corresponding noncancerous mucosae. The average level of DNA demethylase mRNA expression normalized with GAPDH mRNA in stomach cancers was significantly lower than that in the corresponding noncancerous mucosae (Table I, \( p < 0.0001 \)). DNA demethylase mRNA levels normalized with histone H4 mRNA were reduced in 22 (92%) of the 24 stomach cancers when compared with the levels in the corresponding noncancerous mucosae. In 18 patients, levels of DNA demethylase mRNA normalized with histone H4 mRNA in stomach cancers were reduced by 50% or more when compared with the levels in the corresponding noncancerous mucosae. The average level of DNA demethylase mRNA expression normalized with histone H4 mRNA in stomach cancers was significantly lower than that in the corresponding noncancerous mucosae (Table I, \( p < 0.0001 \)). Among stomach cancers, there was no significant association between DNA demethylase mRNA level and clinicopathological parameters of the tumors, e.g., histological differentiation, presence or absence of vascular involvement, and lymph node metastasis.

**DISCUSSION**

This paper is the first to report frequent and significant reduction of DNA demethylase mRNA expression in human cancers. Reduction of DNA demethylase mRNA expression may play a role at a certain step of multistage carcinogenesis. Among the examined cancers, which had no obvious bias to any clinicopathological parameter, the incidence of reduced DNA demethylase mRNA expression was high, and consequently there was no significant association between the DNA demethylase mRNA level and the malignant potential of the tumors. These data suggest that reduction of DNA demethylase mRNA expression may be, if anything, one of the early events of carcinogenesis, but may not participate in the malignant progression of tumors. In addition, studies of precancerous lesions, e.g., adenomas for colorectal cancers, and adenomas and foci showing intestinal metaplasia for stomach cancers, are meaningful when considering the role of this reduction in multistage carcinogenesis. Since reduced expression of DNA demethylase mRNA is observed in both colorectal and stomach cancers, this reduction may be a rather common event during human carcinogenesis, regardless of the affected organ.

With respect to another DNA methylation modifier, DNMT1, elevated mRNA expression is known to result in higher activity of this enzyme in human cancer tissues (35). On the other hand, DNA demethylase activity has been purified only from a human lung cancer cell line, A549 (28, 30). To further confirm the significance of this reduction, the cleavage of a methyl residue from 5-methyl cytosine and its release as methanol should be assessed in cancerous tissues showing reduced expression of DNA demethylase mRNA.

In the MBD2 gene the first in-frame start codon is within the CpG island and the second is located 152 codons downstream, just upstream from the methyl CpG-binding domain. Two proteins, MBD2a and MBD2b, correspond to initiation of translation at the first and second methionine codons, respectively (29). DNA demethylase is identical to MBD2b, and enzymatic activity of its longer MBD2a variant is so far unknown (28). The present mRNA expression analysis was unable to distinguish MBD2a and MBD2b. If functional differences between MBD2a and MBD2b can be further clarified, the expression and/or activity of the two proteins should be examined separately in human cancers.

DNMT1, DNMT2, DNMT3a and DNMT3b, and DNA demethylase have so far been identified as DNA methylation modifiers in human cells. In addition, it has been proposed that indirect mechanisms involving base excision and repair are responsible for removal of methyl groups from DNA during development (36, 37). The activity of human DNA 5-methylcytosine glycosylase catalyzing this indirect DNA demethylation pathway has been detected (37, 38), although the cDNA encoding this enzyme has not been cloned. DNA methylation patterns are determined by the even balance of activities of these multiple DNA methylation modifiers. Therefore, reduced expression of DNA demethylase is not necessarily incompatible with reduction of the global level of DNA methylation (9) which is generally observed in human cancers.

Most biological modifications are reversible, and enzymes can usually catalyze either the forward or the reverse reaction. DNA methylation has so far been considered an exception because removal of a methyl group from DNA must involve cleavage of a carbon-carbon bond, which has been considered an unlikely reaction. Contrary to the conventionally accepted model that DNA methylation is heritable and stable, it is now considered to be a reversible signal similar to other physiological and biochemical modifications, since the identification of DNA demethylase (30). Trans-acting factors, though not yet identified, may alter the accessibility of DNA demethylase to specific sites, e.g., promoter regions of specific genes, resulting in protection of these sites from DNA methylation and modulation of the covalent modification patterns of the genome in response to changing physiological cues in normal cells. Reduced expression and/or activity of DNA demethylase may play a role in production and maintenance of regional DNA hypermethylation, which frequently re-
results in reduced expression of tumor suppressor genes, in human cancers. Molecular mechanisms regulating the function of this newly identified DNA demethylase should be studied further in relation to its association with human carcinogenesis.

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REFERENCES