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Limited up-regulation of DNA methyltransferase in human colon cancer reflecting increased cell proliferation

PETER J. LEE*†, LARAINE L. WASHER†, DAVID J. LAW†, C. RICHARD BOLAND†, ISABELLE L. HORON‡, AND ANDREW P. FEINBERG*†§¶

*Program in Cellular and Molecular Biology, and Departments of †Internal Medicine and §Human Genetics, University of Michigan Medical School, 1150 West Medical Center Drive, Ann Arbor, MI 48109; ‡Maryland Center for Health Statistics, 201 West Preston Street, Baltimore, MD 21201; ¶Departments of Medicine, Oncology, and Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Ross 1064, 720 Rutland Avenue, Baltimore, MD 21205

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ABSTRACT **Epigenetic alterations in the genome of tumor cells have attracted considerable attention since the discovery of widespread alterations in DNA methylation of colorectal cancers over 10 years ago. However, the mechanism of these changes has remained obscure. el-Deiry and coworkers [el-Deiry, W. S., Nelkin, B. D., Celano, P., Yen, R. C., Falco, J. P., Hamilton, S. R. & Baylin, S. B. (1991) *Proc. Natl. Acad. Sci. USA* 88, 3470–3474], using a quantitative reverse transcription–PCR assay, reported 15-fold increased expression of DNA methyltransferase (MTase) in colon cancer, compared with matched normal colon mucosa, and a 200-fold increase in MTase mRNA levels compared with mucosa of unaffected patients. These authors suggested that increases in MTase mRNA levels play a direct pathogenetic role in colon carcinogenesis. To test this hypothesis, we developed a sensitive quantitative RNase protection assay of MTase, linear over three orders of magnitude. Using this assay on 12 colorectal carcinomas and matched normal mucosal specimens, we observed a 1.8- to 2.5-fold increase in MTase mRNA levels in colon carcinoma compared with levels in normal mucosa from the same patients. There was no significant difference between the normal mucosa of affected and unaffected patients. Furthermore, when the assay was normalized to histone H4 expression, a measure of S-phase-specific expression, the moderate increase in tumor MTase mRNA levels was no longer observed. These data are in contrast to the previously reported results, and they indicate that changes in MTase mRNA levels in colon cancer are nonspecific and compatible with other markers of cell proliferation.**

Mammalian DNA methylation is a covalent modification of cytosine, predominantly at CpG dinucleotides, that can repress gene transcription (reviewed in ref. 1). DNA methyltransferase (MTase) stably maintains the pattern of DNA methylation after replication by recognizing and methylating hemimethylated DNA. MTase transfers a methyl group from *S*-adenosylmethionine to the 5 position of cytidine (1).

Widespread alterations in DNA methylation were discovered more than 10 years ago in human colon cancer and other tumors (2, 3). These alterations include a global decrease in 5-methylcytosine content as well as both hypomethylation and hypermethylation of individual genes (reviewed in ref. 4). Four recent observations have greatly enhanced interest in this phenomenon. First, methylation may be related to the high frequency of C to T mutations in human tumor genes (5). Second, some genes have been found to be imprinted, i.e., expressed from a specific parental allele. Many tumors show abnormal imprinting, which can lead to abnormal activation of normally silent alleles of genes for growth factors or epigenetic silencing of growth inhibitory genes (reviewed in ref. 6). This altered imprinting is associated with increased methylation of imprint-specific 300- to 1000-bp CpG "islands" rich in CpG dinucleotides (7, 8). Third, disruption of MTase in knockout mice causes decreased methylation of imprint-specific CpG islands and abnormal imprinting (9), as well as partial protection from tumor development when crossed with *min* mice prone to develop colon tumors (10). Fourth, some tumor suppressor genes show increased methylation and might be silenced epigenetically (11, 12).

el-Deiry and coworkers proposed that a mechanism of altered DNA methylation in colon cancer was increased expression of the MTase gene (13). High MTase activity had been observed earlier in cultured tumor cell lines (14), but el-Deiry and coworkers tested directly for a specific role in human tumors progression by comparing MTase mRNA levels in colon cancers and matched normal mucosa (13). Using a reverse transcription (RT)–PCR assay, these authors observed an 18-fold increase in MTase mRNA levels in colon cancers compared with levels in normal colonic mucosa from the same patients (13). Even more striking, they found a 200-fold increase in median MTase mRNA levels, comparing colon cancers to normal mucosa from patients not suffering from colon cancer (13). They inferred that increased MTase activity both predisposes to and accelerates colon cancer progression (13). The same group later reported a more modest but still significant 5.4-fold increase in DNA MTase enzyme activity in colon cancer compared with activity in normal colonic mucosa (15). They ascribed the difference with the previous study to an overestimate in the RT-PCR assay, posttranscriptional or posttranslational modification, or mixed tissue components in the samples (15). They concluded that increased MTase in colon cancer was real, statistically significant, and not due to increased cell proliferation (15). Schmutte and coworkers have found a 3.7-fold increase in MTase mRNA in colon cancer as measured by RT-PCR (16). They could not determine whether this was biologically significant at the cellular level or simply secondary to increased cell proliferation (16).

Because of concerns that RT-PCR may not be a quantitative measure of gene expression, particularly over the ranges described by el-Deiry and coworkers (13), we developed a sensitive RNase protection assay (RPA), linear over three orders of magnitude, to quantify mRNA levels. The RPA was also more direct as it did not depend on generation of cDNA from RNA or on subsequent amplification by PCR. This assay also allowed simultaneous comparison to several different control mRNA species so that we could distinguish between biologically significant differences and changes occurring secondary to increased cell proliferation. We compared MTase mRNA levels in 12 colon carcinomas to matched normal mucosa from the same patients. In addition, we assayed MTase mRNA levels in 18 additional blinded normal mucosal samples

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Abbreviations: MTase, methyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RPA, RNase protection assay; RT, reverse transcription.

from 6 patients, 3 with cancer and 3 without, to detect any changes in the normal mucosa of cancer patients, as suggested to occur in both of the earlier reports (13, 15).

MATERIALS AND METHODS

Tissues. Colon cancers and normal mucosa from 12 patients were examined by a pathologist at the time of surgery and snap frozen until use. Samples were serially sectioned, and histological analysis was performed on sections adjacent to those studied to exclude contaminating normal tissue. Normal mucosa samples from three noncancer patients and three colon cancer patients were also obtained by colon biopsy, each in triplicate, and the samples were blinded to their origin before analysis. Biopsies were obtained under institutional human subjects guidelines. RNA was isolated as described (7).

RPAs. RPAs were performed using an RPA kit (Ambion, Austin, TX). Simultaneous RPAs were performed in the same tube for each sample, using probes for both MTase and an internal standard. To allow simultaneous RPA with control probes of varying lengths and to examine both ends of the region amplified by RT-PCR by el-Deiry and coworkers (13), we designed the following MTase probes: pMT spanned nucleotides 3134–3455 of MTase in pGEM7, and *in vitro* transcription was performed from the SP6 site in the vector, at the 3′ end of the fragment. The pMT plasmid was cut either at an *Xho*I site in the vector 5′ to the insert, thus generating a 381-nt probe protecting a 322-nt RNA fragment, or at an *Nco*I site at nucleotide 3225, generating a 290-nt probe protecting a 231-nt fragment, both probes giving identical results. An additional MT probe, pMT-Sph, spanned nucleotides 2888–4273 of MTase, in pGEM7, and *in vitro* transcription was performed from the T7 site at the 3′ end of the fragment, after digestion with *Bgl*II, generating a 147-nt probe protecting a 121-nt fragment. A probe protecting a 260-nt fragment of β-actin was included in the kit. The human glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH; a gift of J. Schwartz, University of Michigan, Ann Arbor, MI) was subcloned to generate a probe protecting a 183-nt fragment. The histone H4 gene (a gift of M. Imperiale, University of Michigan, Ann Arbor, MI) was subcloned to generate a probe protecting a 71-nt fragment. RPA was performed essentially as recommended by the manufacturer, using 1–5 μg of total cellular RNA. Electrophoresis was performed in 6% polyacrylamide/8 M urea gels. A 2.1-kb *in vitro* transcript of MTase, including both regions protected by RPA, was synthesized from an MTase cDNA subclone using SP6 RNA polymerase, and serial dilutions were used to generate a standard curve. Quantitation was performed on a Betascope 603 blot analyzer (Betagen, Waltham, MA).

RESULTS

Development of an RPA for MTase. MTase is generally present at low levels except in dividing cells (17). Although detection of the message in Northern blots has been possible with RNA preparations from certain cell lines, quantitative studies involving primary human tumors have been technically difficult, prompting the development of an RT-PCR assay (13) and the RPA described here. This was particularly important in this study, given our approach of dissecting histologically pure sections.

To determine whether the RPA with our MTase probe closely estimated true mRNA levels, we first performed RPA using 0.01–1 μg of purified poly(A)$^+$ tissue mRNA. The same poly(A)$^+$ mRNA preparations were subjected to conventional Northern blot analysis, and the results were compared. RPA using probe pMT was linear over a wide range and produced an excellent approximation of Northern blot analysis (data not shown). However, the RPA required much less RNA than Northern blot analysis, 0.01 μg versus 0.5 μg to accurately estimate MTase mRNA levels, permitting analysis of histologically pure surgical and biopsy specimens.

In addition, considering that el-Deiry and coworkers observed an increase in MTase mRNA levels in excess of 200-fold (13), it was important to establish that the conditions of our assay did not limit the detection of a wide range of MTase RNA. We thus performed RPA using *in vitro* synthesized sense MTase RNA over a 1000-fold dilution. The assay was linear between 0.1 and 350 cpm of protected probe (Fig. 1), well within the range measured during RPA on the tissues studied. Furthermore, had there been a significant increase in MTase RNA in any sample, the assay would have been able to detect it.

MTase mRNA Levels in Paired Colon Cancers and Normal Mucosa. Since small amounts of tissues were analyzed after histological microdissection, the RPA was performed in triplicate on each sample using two probes simultaneously in the same tube, MTase and a second probe to control for RNA loading. Experiments were performed using β-actin, GAPDH, or histone H4 as the second probe. The ratio of MTase to control did not vary significantly for a given sample, even when substantially different amounts of total RNA were loaded (Fig. 2*A*; MTase/β-actin = 1.48, 1.46, 1.47, 1.47; $P < 0.01$).

Using β-actin as the internal standard, six paired colon cancer and normal mucosal samples were first analyzed, each in triplicate. There was on average a slightly increased ratio of MTase mRNA levels in the tumors to MTase mRNA levels in the matched normal mucosa from the same patients (Fig. 2*B*). The ratio of cancer to normal MTase mRNA levels ranged from 0.81 ± 0.36 to 2.96 ± 0.91 (Table 1). The average increase in MTase mRNA levels in the cancers was 1.83 ± 0.38, a small but statistically significant difference ($P < 0.05$, compared with a ratio of 1.0).

Next we analyzed 11 paired cancer and normal samples in triplicate, using GAPDH rather than β-actin as the internal standard. The ratio of MTase mRNA levels in the tumors to those in the matched normal mucosa from the same patients was somewhat greater than that seen using β-actin as the internal standard (Fig. 2*C*). The ratio of cancer to normal

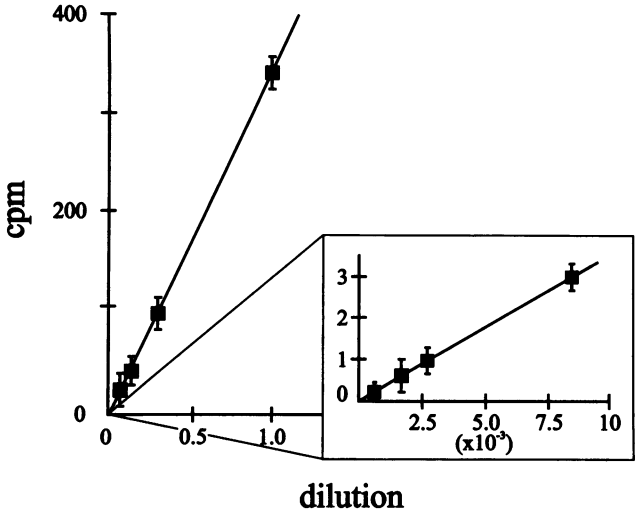

FIG. 1. Linearity of the RPA. *In vitro*-transcribed sense MTase mRNA was gel-purified and subjected to RPA. The *y* axis represents the counts per minute obtained by counting the protected fragments in a Betagen Betascope blot analyzer. The *x* axis represents the dilution of *in vitro* transcribed mRNA subjected to the assay. The maximal amount of *in vitro* transcribed mRNA was 1.7×10^{-3} pg (dilution = 1.0). Each data point represents three to six independent experiments, and the error bars indicate the standard deviation. Counts per minute are shown as over background (<0.1).

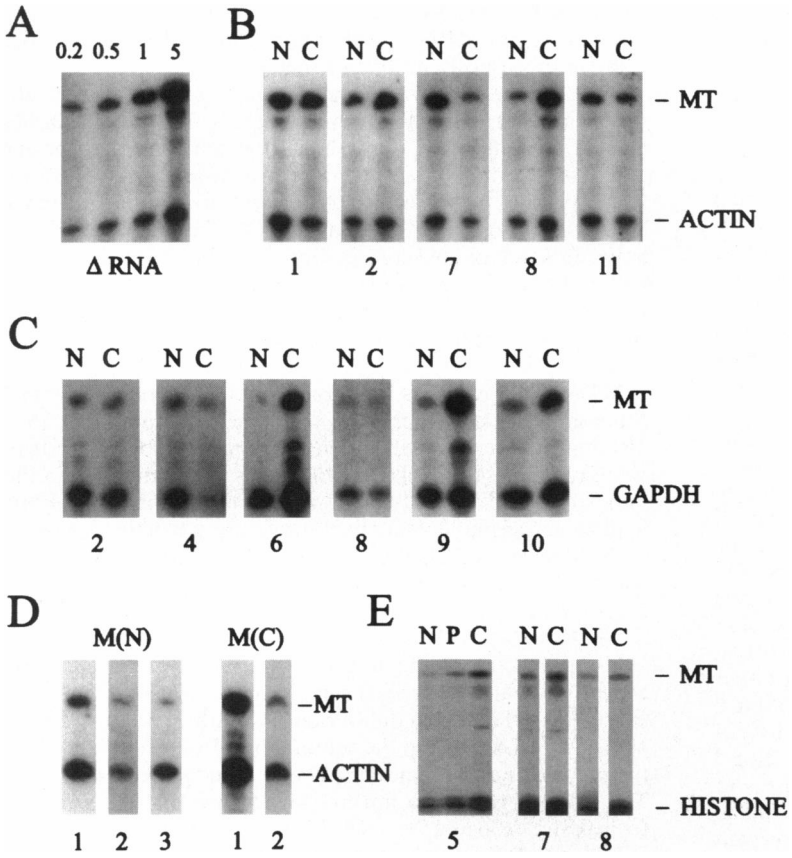

FIG. 2. RPA of MTase in colon cancers and normal mucosa. (*A*) RPA using β-actin as the internal standard and varying amounts of tissue RNA (in micrograms). The assay is linear with respect to the standard. (*B*) RPA of matched normal mucosa (N) and colon cancers (C) from the same patients (numbered), using β-actin as the internal standard. (*C*) RPA using GAPDH as the internal standard. (*D*) RPA of normal mucosa from noncancer [M(N)] and cancer [M(C)] patients, blinded to their origin. (*E*) RPA of matched normal mucosa (N), polyp (P), and cancer (C), using histone H4 as the internal standard.

MTase ranged from 0.83 ± 0.10 to 7.55 ± 0.37 (Table 1). The average fold increase in MTase mRNA levels using GAPDH as the internal standard was 2.57 ± 0.64, again showing a small but statistically significant increase ($P < 0.05$, compared with a ratio of 1.0). Although this ratio was slightly greater than that seen using β-actin as the internal standard, the difference between the ratios using β-actin and GAPDH as the internal standard was not statistically significant. One adenomatous polyp was also examined and showed a slight, 1.37-fold increase in MTase mRNA levels compared with normal mucosa (Table 1).

We considered the possibility that the region of MTase subjected to RT-PCR by el-Deiry and coworkers (13) might contain a region with differential susceptibility to degradation, accounting for the marked differences in our results. Thus we generated an additional MTase probe, pMT-Sph, at the 3′ end of the region amplified by el-Deiry and coworkers (13). The results from use of this probe did not differ significantly from those obtained by use of he more 5′ probe, ranging from 0.97 to 3.10, using β-actin as the internal standard, with an average of 1.71 ± 0.38 (data not shown).

Expression of MTase in Normal Mucosa of Cancer and Noncancer Patients. el-Deiry *et al.* reported increased MTase

Table 1. RPA of DNA MTase expression—matched tissue samples

		MTase-T/MTase-N*		
Patient	Tissue	β-Actin standard	GAPDH standard	Histone standard
1	Cancer/normal	N.D.	0.90 ± 0.07	N.D.
2	Cancer/normal	0.93 ± 0.29	1.07 ± 0.07	0.77 ± 0.01
3	Cancer/normal	2.60 ± 0.22	7.55 ± 0.37	0.67 ± 0.01
4	Cancer/normal	N.D.	2.23 ± 0.22	0.90 ± 0.02
5	Polyp/normal	N.D.	1.37 ± 0.02	1.17 ± 0.01
	Cancer/normal	N.D.	4.28 ± 0.23	1.17 ± 0.01
6	Cancer/normal	N.D.	2.82 ± 0.08	2.84 ± 0.02
7	Cancer/normal	0.81 ± 0.36	1.45 ± 0.12	0.84 ± 0.01
8	Cancer/normal	2.39 ± 1.00	1.23 ± 0.05	N.D.
9	Cancer/normal	2.96 ± 0.91	4.72 ± 0.24	N.D.
10	Cancer/normal	N.D.	1.22 ± 0.09	0.72 ± 0.02
11	Cancer/normal	1.27 ± 0.18	N.D.	N.D.
12	Cancer/normal	N.D.	0.83 ± 0.10	N.D.
Average	Cancer/normal	1.83 ± 0.38 ($P < 0.05$)†	2.57 ± 0.64 ($P < 0.05$)†	1.17 ± 0.30 (N.S.)

N.S., not statistically significant; N.D., not done.
*Ratio of tumor MTase mRNA levels to matched normal mucosa MTase mRNA levels, relative to the internal standard.
†The probability (*P*) that the ratio differs from 1.0 is less than 0.05.

Table 2. RPA of DNA MTase expression—blinded mucosal biopsies

Noncancer patient	Tissue	MTase/ β-actin*	Cancer patient	Tissue	MTase/ β-actin*
1N	Normal mucosa	0.85 ± 0.31	4N	Normal mucosa	0.24 ± 0.03
2N	Normal mucosa	0.45 ± 0.11	5N	Normal mucosa	0.18 ± 0.06
3N	Normal mucosa	0.29 ± 0.12	6N	Normal mucosa	0.38 ± 0.07
Average	Normal mucosa (Noncancer patients)	0.53 ± 0.29	Average	Normal mucosa (Cancer patients)	0.27 ± 0.10

*MTase mRNA levels, normalized to internal β-actin standard, in relative units.

mRNA levels in the normal mucosa of cancer patients, compared with the normal mucosa of noncancer patients (13). A subsequent study by the same group also reported a statistically significant 5.4-fold increase in MTase enzyme activity in the normal mucosa of cancer patients (15), even though this was less marked than the previously reported increase in gene expression (13). Therefore, we measured the MTase mRNA levels in fresh colonoscopic biopsies, comparing the normal mucosa of three patients without neoplasia to three patients with cancer. These assays were done on three independent biopsy specimens from each patient. To further eliminate any possible experimental bias in the assay, all 18 samples were blinded for their source. There was no increase in MTase mRNA levels in the normal mucosal specimens from cancer patients, compared with the normal mucosa of noncancer patients, or compared with the normal mucosa samples from the cancer patients analyzed previously (Fig. 2*D* and Table 2). Indeed, the level in normal mucosa was somewhat lower in the cancer patients than in the noncancer patients, although this difference was not statistically significant (Table 2). In summary, MTase mRNA levels was increased an average of 1.83-fold (β-actin standard) to 2.57-fold (GAPDH standard) in colon cancers above that seen in normal mucosa of the same patients, but it was not increased in the normal mucosa of colon cancer patients compared with patients without cancer.

MTase mRNA Levels Normalized to Histone H4. As MTase expression is increased in proliferating cells (17), the observed increases in MTase mRNA levels in tumor tissues might simply be the result of increased cell proliferation in the cancers. To test this hypothesis, we performed additional RPAs using as the internal standard histone H4, a marker of S-phase-specific gene expression (18). These experiments revealed that MTase mRNA levels in colon cancer, normalized to histone H4 expression, was not significantly different from that of normal mucosa (Fig. 2*E*). The ratio of MTase mRNA in the tumors to that of matched normal mucosa of the same patients ranged from 0.67 ± 0.01 to 2.84 ± 0.02, with an average of 1.17 ± 0.30 (not significantly different than 1.0). Therefore, the observed increase in MTase mRNA in colon cancers can be explained by an increased rate of cell proliferation in the tumors.

An intermediate level of MTase in adenomas had been reported in both of the earlier reports (13, 15), and it was concluded that MTase is specifically linked to stepwise progression in colon cancer. However, RNA from normal, polyp, and cancer of the same patient showed a progressive increase in MTase mRNA, but identical to the increase in histone H4 expression analyzed concurrently (Fig. 2*E* and Table 1). This result is not surprising, given that there was no observed increase in MTase mRNA levels in colon cancer compared with matched normal mucosa, when normalized to histone H4 expression (Fig. 2 and Table 1).

DISCUSSION

In summary, using a sensitive and linear RPA, we found that MTase mRNA levels are only modestly elevated in colon cancer, an average of 1.83- to 2.57-fold. These data are in contrast to the 15- to 20-fold increase originally reported by el-Deiry and coworkers (13) or to the 5.4-fold elevation in MTase activity later reported by the same group (15). Furthermore, the difference between cancer and normal disappeared when the internal standard was histone H4, a measure of cell proliferation. In addition, no significant differences were seen in MTase mRNA comparing the normal mucosa of patients with colon cancer to that of noncancer patients, in contrast to the 15-fold increase reported by el-Deiry and coworkers (13) and the 1.6-fold increase in enzyme activity reported by Issa and coworkers (15).

What could account for the differences between these two studies? Most likely, the quantitative RT-PCR assay used by el-Deiry and coworkers (13) was not linear, or the large 1.2-kb fragment subjected to RT-PCR by that group was differentially degraded in the tumor and normal tissues of paired specimens. Normal colonic mucosa undergoes rapid autolysis after removal, and it is also less cellular and yields less RNA per volume than colon cancers. In addition, a noncompetitive rather than competitive internal standard was used in that study (13), which can also lead to significant variability (19). The comparatively low level of MTase mRNA levels in tumors observed in the present study was also not due to inadvertent contamination with normal tissue, as parallel sections were histologically examined, and analysis was done only on regions of >95% histological purity. Alternatively, we may have examined a different patient subpopulation than did el-Deiry and coworkers (13) and Issa and coworkers (15). However, our samples included both early and advanced stage tumors from both proximal and distal colon.

Our data are also consistent with a recent study by Schmutte and coworkers (16), who found a 3.7-fold elevation in MTase mRNA in colon cancer compared with normal colonic mucosa. The latter study, like that of el-Deiry and coworkers (13), used a quantitative RT-PCR assay, but with amplification over a smaller portion of the gene. We did not examine MTase enzymatic activity in colon specimens, although based on the previous reports, one would expect less of a difference than seen for gene expression (12, 14). Furthermore, our data and those of Schmutte and coworkers (16) are consistent with the reported difference in MTase activity (15). However, as noted by Schmutte and coworkers, none of the previous studies could distinguish between an acute increase in MTase mRNA and an increase in the proportion of dividing cells (16). The present study clearly implicates increased cell proliferation as the cause of the apparent increase in MTase mRNA in colon tumors.

Do our results exclude a direct role for MTase in carcinogenesis? Not necessarily, as there could be a change in the regulation of MTase activity or on its gene target specificity, without alteration in the level of expression. Several groups have observed increased methylation of normally unmethylated CpG islands in tumors (20–22). We and others have observed aberrant increased methylation of CpG islands, which appear to mark imprinted genes associated with loss of genomic imprinting in cancer (7, 8). However, we believe that if MTase plays a role in this process, it is not mediated by an increase in gene expression but involves more subtle changes, such as posttranslational modification and/or changes in subcellular localization (23).

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