

• BRIEF REPORTS •

# Hypermethylation of CpG island in O<sup>6</sup>-methylguanine-DNA methyltransferase gene was associated with *K-ras* G to A mutation in colorectal tumor

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## Abstract

**AIM:** To investigate the functions of promoter hypermethylation of O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) gene in colorectal tumorigenesis and progression.

**METHODS:** The promoter hypermethylation of MGMT gene was detected in 27 sporadic colorectal adenomas, 62 sporadic colorectal carcinomas and 20 normal colorectal mucosa tissues by methylation-specific PCR. At the same time, the expression of MGMT protein was carried out in the same samples using immunohistochemistry. Mutant-allele-specific amplification was used to detect *K-ras* G to A point mutation in codon 12.

**RESULTS:** None of the normal colorectal mucosa tissues showed methylated bands. Promoter hypermethylation was detected in 40.7% (11 of 27) of adenomas and 43.5% (27 of 62) of carcinomas. MGMT proteins were expressed in nucleus and cytoplasm of normal colorectal mucosa tissues. Loss of MGMT expression was found in 22.2% (6 of 27) of adenomas and 45.2% (28 of 62) of carcinomas. The difference between them was significant ( $P = 0.041$ ). In the 6 adenomas and 28 carcinomas losing MGMT expression, 5 and 24 cases presented methylation, respectively ( $P = 0.027$ ,  $P < 0.001$ ). Thirteen of the 19 colorectal tumors with *K-ras* G to A point mutation in codon 12 had methylated MGMT ( $P = 0.011$ ). The frequencies of *K-ras* G to A point mutation were 35.3% (12 of 34) and 12.7% (7 of 55) in tumors losing MGMT expression and with normal expression, respectively.

**CONCLUSION:** Promoter hypermethylation and loss of expression of MGMT gene were common events in colorectal tumorigenesis, and loss of expression of MGMT occurs more frequently in carcinomas than in adenomas in sporadic patients. Hypermethylation of the CpG island of MGMT gene was associated with loss of MGMT expression and *K-ras* G to A point mutation in colorectal

tumor. The frequency of *K-ras* G to A point mutation was increased in tumors losing MGMT expression. It suggests that epigenetic inactivation of MGMT plays an important role in colorectal neoplasia.

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**Key words:** O<sup>6</sup>-methylguanine-DNA methyltransferase; CpG island; DNA methylation; Epigenetic change; *K-ras* mutation

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## INTRODUCTION

O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) is a ubiquitous DNA repair protein that removes mutagenic and cytotoxic adducts from the O<sup>6</sup>-guanine in DNA, the preferred point of attack of many carcinogens and alkylating chemotherapeutic agents<sup>[1,2]</sup>. Alkylating of DNA at the O<sup>6</sup> position of guanine is an important step in the formation of mutations in cancer, primarily due to the tendency of the O<sup>6</sup>-methylguanine to pair with thymine during replication, resulting in a conversion of guanine-cytosine to adenine-thymine pairs in DNA<sup>[3]</sup>. Furthermore, the O<sup>6</sup>-alkylguanine-DNA adduct may cross-link with the opposite cytosine residues, blocking DNA replication<sup>[4-6]</sup>. MGMT expression is decreased in some tumor tissues, and lack of activity has been observed in some cell lines<sup>[7,8]</sup>. Loss of expression is rarely due to genetic changes of the MGMT gene, but methylation of discrete regions of the CpG island of MGMT has been associated with the silencing of the gene in cell lines<sup>[9,10]</sup>. *K-ras* mutation occurs in approximately one-half of colorectal carcinomas. Alteration of codon 12 GGT is the most common change, and often results from G to A transitions. In this study, we examined whether the epigenetic silencing of MGMT might be linked to the presence of *K-ras* mutations in human colorectal tumorigenesis.

## MATERIALS AND METHODS

### Materials

Twenty-seven sporadic colorectal adenomas and 62 sporadic carcinomas were obtained from surgical resection specimens of surgical patients at the Zhongnan Hospital of Wuhan

University between May 2002 and June 2003. Twenty normal colorectal mucosa tissues were obtained from healthy volunteers examined by endoscopy in the clinic. Each sample was divided into two sections: one was stored at  $-70^{\circ}\text{C}$ , the other was fixed in formalin solution, embedded by paraffin until processing. No patient had received chemotherapy or radiation therapy prior to surgery. Genomic DNA was extracted by the phenol–chloroform standard methods.

## Methods

**Methylation-specific PCR (MSP)** Bisulfite genomic DNA modification was performed as previously reported<sup>[11]</sup>. Genomic DNAs purified from tissues were denatured by NaOH and modified by sodium bisulfite, then purified using Wizard DNA purification resin (Promega), again treated with NaOH, precipitated with ethanol, and resuspended in water. PCR amplification was performed to determine the DNA methylation patterns in the CpG island of the *MGMT*. Primer sequences of *MGMT* were for the unmethylated reaction 5'-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3' (sense primer), 5'-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3' (antisense primer) and for the methylated reaction 5'-TTT CGA CGT TCG TAG GTT TTC GC-3' (sense primer), 5'-GCA CTC TTC CGA AAA CGA AAC G-3' (antisense primer). Amplifications were performed in 50  $\mu\text{L}$  of reaction mixture under the following conditions: 1 cycle at  $95^{\circ}\text{C}$  for 360 s, 35 cycles at  $94^{\circ}\text{C}$  for 45 s,  $59^{\circ}\text{C}$  45 s, and  $72^{\circ}\text{C}$  for 60 s; and a final extension at  $72^{\circ}\text{C}$  for 600 s. Amplified products were visualized by 8% polyacrylamide gel electrophoresis.

**Mutant allele-specific amplification (MASA)** G to A mutations at codon 12 (GGT) were detected by MASA<sup>[12]</sup>. The 3'-terminal bases of sense primers were set to the mutant base of *K-ras* codon 12; the sense-1 primer was for the first base G to A mutation and the sense-2 primer was for the second. The primer sequences were sense-1, 5'-ACTTGTGGTAGTTGGAGCTA-3'; sense-2, 5'-CTTGTG GTAGTTGGAGCTGA-3'. The antisense primer for both senses was 5'-CTCATGAAAATGGTCAGAGAAACC-3'. Reaction conditions were 1 cycle at  $94^{\circ}\text{C}$  for 300 s, 35 cycles at  $94^{\circ}\text{C}$  for 30 s,  $63.5^{\circ}\text{C}$  for 90 s, and  $72^{\circ}\text{C}$  for 90 s and a final extension at  $72^{\circ}\text{C}$  for 300 s. Amplified products were visualized by 8% polyacrylamide gel electrophoresis.

**Immunohistochemistry** Formalin-fixed, paraffin-embedded samples were cut into 4- $\mu\text{m}$  sections. Immunohistochemistry was carried out using a SP kit and mouse anti-*MGMT* monoclonal antibody. Nuclear staining was counted as positive. Those cases with lesser than 10% positively stained cells were regarded as having loss of expression. The stain was assessed by a pathologist.

## Statistical analysis

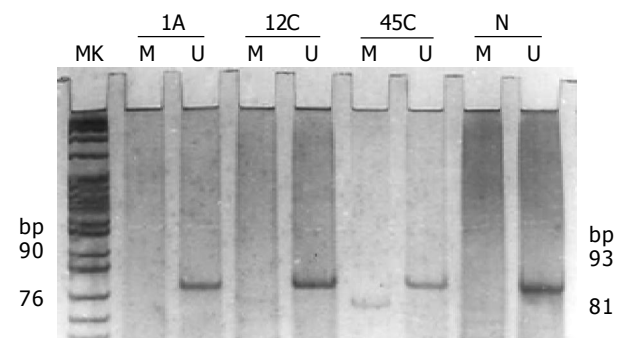
Statistical analysis was performed using SPSS 11.0 software. Associations between the discrete variables were assessed using the two-sided Fisher's exact test or Pearson's  $\chi^2$  tests. *P* value less than 0.05 was regarded as statistically significant.

## RESULTS

### *MGMT* promoter hypermethylation

DNA obtained from 89 colorectal tumors and 20 normal

colorectal mucosa tissues was subjected to *MGMT* promoter methylation study using MSP. None of the normal tissues showed methylated bands (81 bp), but unmethylated bands (93 bp). Promoter hypermethylation was detected in 38 of 89 (42.7%) colorectal tumors (Figure 1). Among the adenomas and carcinomas tested, 11 of 27 (40.7%) and 27 of 62 (43.5%) had promoter hypermethylation, respectively. No statistical difference could be found between them ( $\chi^2 = 0.061$ ,  $P = 0.806$ ). But when we compared the promoter hypermethylation status of colorectal adenomas and carcinomas with normal colorectal mucosa tissues, the percentages of them were both higher than that of the normal tissues (Fisher's exact test,  $P = 0.001$ ,  $P < 0.001$ ). Aberrant *MGMT* promoter hypermethylation was frequent in both sporadic colorectal adenomas and carcinomas.

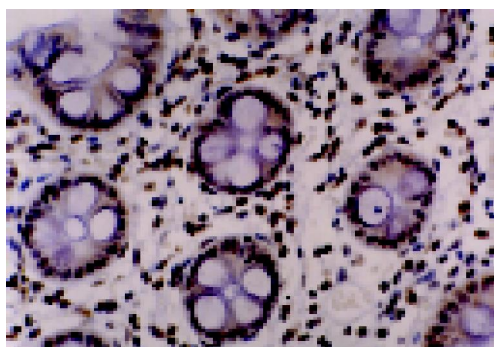


**Figure 1** Methylation-specific PCR result of *MGMT* in colorectal tumors and normal colorectal mucosa. Colorectal carcinomas (45 °C) showed both unmethylated (U) and methylated (M) bands, but normal tissues (N) showed only unmethylated bands.

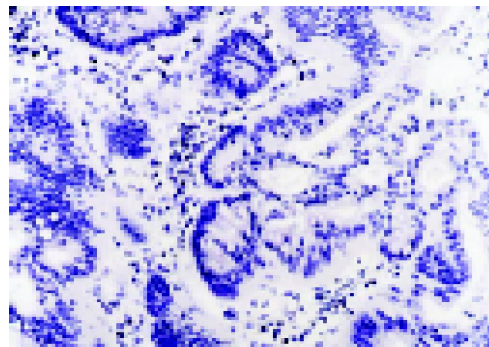
### Expression of *MGMT*

We analyzed *MGMT* expression by immunohistochemistry so that the expression in the neoplastic cells could be directly measured. *MGMT* protein was normally expressed in the nucleus of most parenchymal and stromal cells (Figure 2). All normal colorectal mucosa tissues examined had nuclear staining of *MGMT*. Among 89 colorectal tumors analyzed, 34 were judged to have loss of *MGMT* expression, whereas 55 tumors expressed *MGMT* (Figure 3). In these cases, 6 of 27 (22.2%) adenomas and 28 of 62 (45.2%) carcinomas showed loss of *MGMT* expression. Therefore, carcinoma significantly differs from adenoma in terms of *MGMT* expression ( $\chi^2 = 4.192$ ,  $P = 0.041$ ). And also colorectal adenomas and carcinomas both differ from normal colorectal mucosa tissues (Fisher's exact test,  $P = 0.031$ ,  $P < 0.001$ ).

When the expression data were matched to previous studies of the methylation of *MGMT*, 5 of 6 adenomas that lack *MGMT* expression showed *MGMT* promoter hypermethylation, whereas 15 of 21 adenomas that retained expression of *MGMT* were unmethylated at the *MGMT* CpG island (Fisher's exact test,  $P = 0.027$ ); 24 of 28 carcinomas lacking *MGMT* expression showed *MGMT* promoter hypermethylation, whereas 31 of 34 carcinomas that retained expression of *MGMT* were unmethylated at the CpG island ( $\chi^2 = 36.927$ ,  $P < 0.001$ ). It suggests that the presence of aberrant hypermethylation in *MGMT* promoter was significantly associated with loss of *MGMT* protein.



**Figure 2** MGMT expressed in the nuclei of normal colorectal mucosa cells.



**Figure 3** Loss of MGMT expression in colorectal carcinomas.

### ***K-ras* G to A point mutation in codon 12**

Among 89 colorectal tumors, 19 (21.3%) had *K-ras* G to A point mutation in codon 12 (Figure 4). This event was associated with the promoter hypermethylation of *MGMT*, for 13 (68.4%) of the 19 tumors with mutant *K-ras* had methylated *MGMT*, whereas only 25 (35.7%) of the 70 tumors without *K-ras* mutations had methylated *MGMT* ( $\chi^2 = 6.534$ ,  $P = 0.011$ ).

In the tumors that had loss of MGMT expression, the G to A point mutation rate of *K-ras* at codon 12 was 35.3% (12 of 34). This was 2.8 times greater than the rate 12.7% (7 of 55) in the tumors with normal MGMT expression ( $\chi^2 = 6.373$ ,  $P = 0.012$ ).

## **DISCUSSION**

Gene function in cancer can be disrupted either through genetic alterations, which directly mutate or delete genes, or epigenetic alterations, which alter the heritable state of gene expression. It is increasingly apparent that, in human cancers, synergy between these two processes drives tumor progression from the earliest to latest stages. Methylation is the main form of epigenetic change in humans, and alterations in methylation patterns play an important role in tumorigenesis<sup>[1]</sup>. In this report, we provide an example of the interaction between epigenetic and genetic events in human cancer.

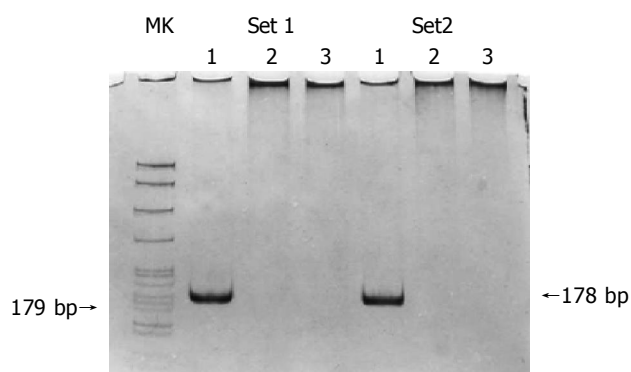
Our data showed that *MGMT* promoter hypermethylation

and loss of MGMT expression were both frequent in sporadic colorectal tumors. This is conformed by the previous report<sup>[13]</sup>. Loss of expression is not commonly due to deletion, mutation or rearrangement of the *MGMT* gene, or mRNA instability, but due to methylation of the CpG island of *MGMT*<sup>[9,14]</sup>. In cell culture, a relationship between methylation of the *MGMT* CpG island and transcriptional silencing of the gene has been demonstrated<sup>[9]</sup>. Our study demonstrates that *MGMT* promoter hypermethylation associated with loss of expression is frequent in sporadic colorectal tumorigenesis as well. This tight correlation between *MGMT* CpG island methylation and loss of expression in these tumors provides an explanation for the loss of MGMT activity in earlier and our current reports.

We also found that *MGMT* promoter hypermethylation was present in equal frequency in adenomas and carcinomas, but the rate of loss of MGMT expression was higher in carcinomas than in adenomas, which suggests that *MGMT* methylation occurs early in colorectal neoplastic progression, and methylated *MGMT*, DNA repair gene in the adenoma stage resulted in the inactivation of *MGMT*, as it brings about the loss of MGMT protein expression when adenoma transforms into carcinoma. Therefore, *MGMT* methylation might play a key role in malignant transformation.

*MGMT* is a key enzyme in the DNA repair network. *MGMT* removes alkyl adducts from the O<sup>6</sup> position of guanine, and then inactivates the proliferation of cells with damaged DNA. Therefore, it can protect cells from alkylating damages and maintain genomic integrity<sup>[15]</sup>. Our results suggest that the epigenetic alteration in *MGMT* promoter may confer an increased risk of alkylating agent-induced colorectal carcinogenesis.

Alkylating agents causing the promutagenic lesion may be provided from dietary nitrates reduced in the proximal colon by bacteria, by nitrosation of amines and amides derived of protein catabolism<sup>[16,17]</sup>. During DNA replication, O<sup>6</sup>-methylguanine can pair with thymine, resulting in conversion of guanine–cytosine to adenine–thymine pairs in DNA<sup>[3]</sup>. Supporting these data, the most common mutations caused by alkylating agents are G:C to A:T transitions. *K-ras* mutation occurs in approximately one-half of colorectal carcinomas. *Ras* oncogenes acquire transforming activity following single-point mutations within their coding sequences. Mutations in naturally occurring *ras* oncogenes have been localized in codons 12, 13, 59 and 61. Alteration



**Figure 4** MASA-PCR products for detection of *K-ras* point mutations in colorectal tumors. Set 1 and set 2 showed the first and second G to A mutation in *K-ras* codon 12 (GGT), respectively.

of codon 12, GGT, is the most common change. Substitution of the wild-type glycine 12 by any other amino acid results in oncogenic activation of this molecule. Previous studies had suggested that loss of *MGMT* activity in the normal colorectal mucosa was found in patients with G to A mutations in *K-ras*<sup>[18,19]</sup>. However, our study suggests that epigenetic silencing of the DNA repair gene *MGMT* by hypermethylation is strongly associated with G to A mutations in *K-ras* in colorectal tumorigenesis. On the other hand, the absence of *K-ras* mutations is a common feature of sporadic human breast carcinomas. Concordant with these phenomena, *MGMT* is not inactivated by promoter hypermethylation in breast tumors<sup>[4]</sup>. These suggest that one potential consequence of loss of *MGMT* expression could be an increase in susceptibility of G to A mutation in *K-ras* gene.

In conclusion, our studies show loss of *MGMT* expression via hypermethylation in *MGMT* promoter leading to activation of *K-ras* gene by accumulation of G to A transition is a new potential pathway of colorectal tumorigenesis. Otherwise, our data concerns the fact that each guanine in the human genome may generate a promutagenic lesion in the absence of correct repair. Other genetic alterations and mutations in target genes in human cancer that might be induced by *MGMT* epigenetic inactivation should receive close attention, and be investigated in the future.

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