Reduced Expression Level of *Mgmt* mRNA and *β-Catenin* Gene Mutation in Rat Colon Tumors

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Abstract. *Background: O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein and protects DNA from the biological effects of alkylating carcinogens. The purpose of this study was to investigate the association between the mRNA expression level of the Mgmt gene and mutation of the β-catenin gene in rat colon tumors induced by azoxymethane (AOM) plus dextran sulfate sodium (DSS). Materials and Methods: Eleven tumor samples from rat colon treated by AOM plus DSS were examined. Mutation of the β-catenin gene was identified by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis. The expression level of Mgmt mRNA was determined by reverse transcription-PCR (RT-PCR). Results: Four out of five adeno-carcinoma samples bearing β-catenin gene mutation (5 out of 11, 45%) displayed a decrease in expression levels of Mgmt mRNA ($p<0.02$). Conclusion: These results suggest that the reduced expression of Mgmt mRNA and β-catenin gene mutation may contribute to the development of rat colon tumors.*

O^6-methylguanine-DNA methyltransferase (MGMT) is one of the key enzymes that protect DNA from the biological effects of alkylating agents (1). Alkylating agents such as azoxymethane (AOM) and dimethylhydrazine (DMH) are representative carcinogens of the murine colon and cause the formation of the O^6-methylguanine (O^6-MeG) adduct in the DNA strand (2). Once the O^6-MeG adduct is formed, guanine can only pair with thymidine during DNA replication, eventually resulting in a G:C to A:T transition mutation (3). Therefore, the MGMT protein is considered to play an important role in the carcinogenesis process (4). Zaidi *et al.* found that, in MGMT transgenic mice, overexpression of the human MGMT protein caused a significant reduction in both the aberrant crypt foci (ACF) formation and the frequency of *K-ras* gene mutation in the ACF (5). In addition, Wali *et al.* reported that O^6-benzylguanine, a specific inhibitor of the MGMT protein, increased the development of colon tumor and the incidence of *K-ras* gene mutation in an AOM-treated rat model (6). An association between the expression of *MGMT* mRNA and G:C to A:T transition mutation in colon cancer surgical specimens has also been suggested (7).

β-Catenin gene mutations have been found in both sporadic human colon cancer (8) and carcinogen-induced murine colon tumors (9, 10). The G:C to A:T transition mutation is also frequently found in the *β-catenin* gene in carcinogen-induced rat colon tumors (9, 10). In a recent study, we found G:C to A:T transition mutations of this gene in DMH/dextran sulfate sodium (DSS)-induced rat colon tumors (11). However, the association between *MGMT* mRNA expression and *β-catenin* gene mutation has not yet been elucidated. In the present study, the association between the expression level of Mgmt mRNA and mutation of *β-catenin* gene in AOM/DSS-induced colon tumors was examined.

Materials and Methods

Animal treatment and tissue collection. To induce colon tumors, five-week-old male F344 rats were given subcutaneous injections of AOM (20 mg/kg body weight, Sigma Chemical Co., St. Louis, MO, USA) twice a week for 1 week, as described previously (11). Briefly, 1 week after the first injection of AOM, the rats were given 1% DSS (ICN Biomedicals Inc., Aurora, OH, USA) in drinking water for 1 week and were then maintained on tap water to the end of the experiment. All the animals were sacrificed under CO_2 anesthesia at 11 or 15 weeks after the start of the experiment. All of the identified colon tumors were carefully removed and cut into two pieces. One piece was fixed in 10% buffered formalin for histological examination and the second

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piece was assessed for reverse transcription polymerase chain reaction (RT-PCR) and PCR single-strand conformation polymorphism (SSCP) analyses. The rats were maintained in the Animal Facility of the University of the Ryukyus, Japan, according to the Institutional Animal Care Guidelines.

RT-PCR analysis. These assays were performed by established procedures (12). In brief, total RNA was extracted from colon tumors using the ISOGEN reagent (NIPPON GENE Co, Ltd., Toyama, Japan), as recommended by the manufacturer. Colon tumors were lysed in 1 ml of ISOGEN and the aqueous phase containing RNA was isolated from the organic phase. The RNA was then precipitated with isopropanol, washed with 75% ethanol, dried and dissolved in diethylpyrocarbonate-treated distilled water. cDNA was amplified from 1 μg total RNA using the SuperScript II reverse transcriptase (Invitrogen Co., Carlsbad, CA, USA) and was then amplified by PCR using the following primer sets: primers used for the rat *Mgmt* were 5'-GCC AGA GGG AGT GCC AGA GC-3' and 5'-CGG CTG TCC AGT TGG GAT GC-3', producing a product of 388-bp and primers used for the rat *β-actin* were 5'-GGC TGT GTT GTC CCT GTA TG-3' and 5'-CAC GCA CGA TTT CCC TCT CA-3', producing a product of 221-bp. The amplification consisted of an initial denaturation of 2 min at 94˚C, followed by 40 cycles of 94˚C for 30 sec, 65˚C for 30 sec, 72˚C for 1 min for *Mgmt*, or 23 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 1 min for *β-actin* and a final extension at 72˚C for 5 min. PCR products were visualized on 2% agarose gel with ethidium bromide. *β-Actin* was used to normalize the data and the experiments were repeated to confirm the results. All amplification procedures included RT-free controls consisting of the amplification cocktail, RNA samples and distilled water in place of RT to check for contamination of the RNA samples with DNA.

PCR-SSCP analysis. PCR-SSCP analysis was performed as described previously (11). Briefly, 50-100 mg of the frozen tissues were homogenized in 400 μl of 50 mM SEDTA (pH 8.0). After proteinase K treatment, DNA was extracted with phenol/chloroform and precipitated with 100% ethanol. DNA samples from colon tumors were then PCR-amplified using the following primer sets: primers for the rat *β-catenin* gene were 5'-GGA GTT GGA CAT GGC CAT GG-3' and 5'-TCC ACA TCC TCT TCC TCA GG-3'. These primers were designed to produce 150-bp fragments of the rat *β-catenin* gene exon 3, corresponding to possible phosphorylation sites by glycogen synthase kinase 3β (GSK3β). The amplification cycle consisted of 0.5 min at 94˚C for denaturation, 0.5 min at 55˚C for primer annealing and 1 min at 72˚C for extension. PCR was conducted for 35 cycles in a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The resultant PCR products were applied to a 12% polyacrylamide gel (GeneGel Excel 12.5/24 Kit, Amersham Biosciences, Uppsala, Sweden) at a constant power of 15W and the gel was subjected to silver staining (PlusOne™ DNA Silver Staining Kit, Amersham Biosciences). DNA obtained from the normal liver of an untreated F344 rat was used as a negative control. Tumor samples with the *β-catenin* gene mutation found in previous studies were used as positive controls (10, 13).

Statistical analysis. Differences in expression levels of *Mgmt* mRNA between tumor and normal mucosa were analyzed using ANOVA followed by Dunnett's test. Association between the changes in expression level of *Mgmt* mRNA and the existence of the *β-catenin* gene mutation were analyzed by Fisher's exact probability test. Statements of significance are $p<0.05$.

Results and Discussion

A total of 33 adenocacinomas were induced in the colon. Of these, eleven adenocarcinomas were randomly chosen and used for the following analyses. *β-Catenin* gene mutations were identified by comparing shifted bands in the SSCP gel with those of positive controls, as described in a previous paper (10). *β-Catenin* gene mutations were found in 5 out of 11 (45%) colon tumors (Figure 1a). Of these 5 mutations, 3 (60%) were $^{32}G \rightarrow A$ (first position) and 2 (40%) were $^{34}G \rightarrow A$ (second position).

A total of 11 adenocarcinomas (the same samples used in the SSCP analysis) and 4 normal colon mucosa tissues adjacent to these adenocarcinomas were assessed for RT-PCR assays to determine the expression levels of *Mgmt* mRNA. The expression levels of *Mgmt* mRNA in the tumors were evaluated by comparing those of the normal colon mucosa treated with AOM/DSS. Of these, 4 out of 5 (80%) adenocarcinomas bearing the *β-catenin* gene mutation displayed a significant decrease in expression levels of *Mgmt* mRNA (Figure 1c, T6, T7, T10 and T11, $p<0.03$), whereas 6 out of 6 (100%) adenocarcinomas without *β-catenin* gene mutations showed no significant decrease in expression levels of *Mgmt* mRNA (Figure 1c, T1, T3, T4, T5, T8 and T9). One colon tumor, that had mutation at codon 32, showed no significant decrease in expression level of *Mgmt* mRNA (Figure 1c, T2). *β-Catenin* gene mutations were significantly associated with decreased expression levels of *Mgmt* mRNA ($p<0.02$).

In the present study, it was found that the expression level of *Mgmt* mRNA was reduced in 4 out of 11 rat colon adenocarcinomas induced by AOM/DSS compared to that of the normal mucosa and that these 4 adenocarcinomas harbored G:C to A:T transition mutations of the *β-catenin* gene in rat colon carcinogenesis induced by AOM/DSS. A major adduct, O^6-MeG, was identified in the DNA strand of rats treated with AOM (4). O^6-MeG was also demonstrated to be responsible for tumor induction in carcinogen-treated rats (14). Although O^6-MeG was detected in major organs including the liver, intestine and kidney of rats treated with an alkylating agent (15), tumor was preferentially induced in the colon. The number of O^6-MeG that can be repaired is equal to the number of active Mgmt molecules (4). DMH treatment leads to a transient loss in Mgmt activity in the colon (16). Once Mgmt protein removes alkylating adducts from DNA, the alkylated form of the Mgmt protein becomes unstable and is rapidly degraded, resulting in exhaustion of the Mgmt protein in the colon (4). DNA synthesis consistently results

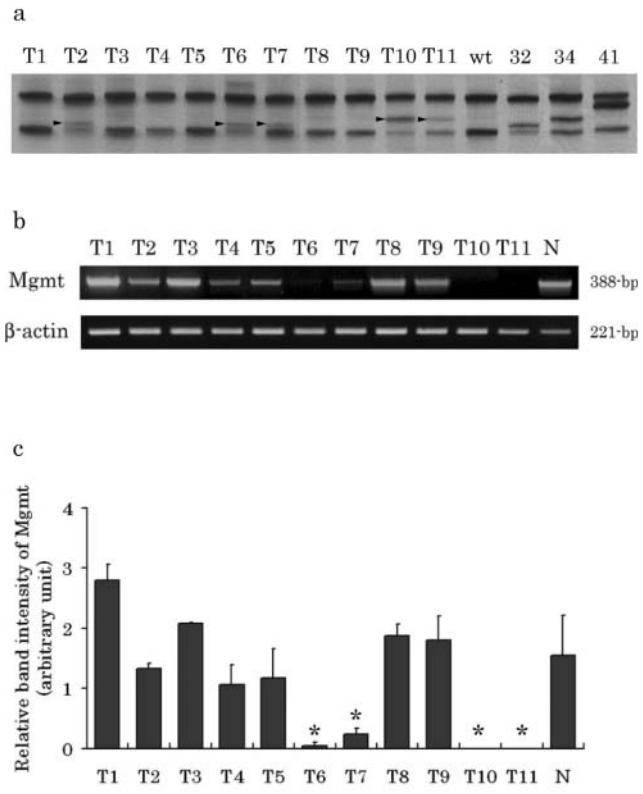

Figure 1. *Mutations of the β-catenin gene and expression levels of Mgmt mRNA. Representative results of PCR-SSCP (a) and RT-PCR (b) analyses are shown. T1 - T11, tumor samples; N, normal colon mucosa treated with AOM/DSS; wt, negative control sample obtained from an untreated F344 rat; 32, 34 and 41, positive control DNA samples at codons 32 (G → A, first position), 34 (G → A, second position) and 41 (C → T, second position), respectively (14). Samples T1 - T4 and T5 - T11 were obtained at 11 and 15 weeks after the start of the experiment, respectively. The band intensity was quantified by densitometric scanning and normalized by β-actin (c). Note the decreased expression levels of Mgmt mRNA in samples T6, T7, T10 and T11 (asterisks) compared to the normal mucosa (N) and band shifts in all these samples (arrowheads). Sample T2 showed band shift but the difference in expression level of Mgmt mRNA was not significant between this sample and normal mucosa (N). Mutation of the β-catenin gene was associated with the decreased expression status of Mgmt mRNA ($p<0.02$).*

in the fixation of mutations at the sites of DNA adducts that have persisted (16). These findings suggest that the expression levels of *MGMT* mRNA may be associated with the sensitivity of organs to the specific carcinogens such as AOM and DMH and also with its ability to repair DNA.

β-Catenin gene mutation leads to constitutive activation of the Wnt pathway and eventually causes an increase in cell proliferation (8). *β-Catenin* gene mutations at codons 32, 34 and 41 are frequently found in rat colon carcinogenesis models (9, 17). The G:C to A:T transition mutations are also frequently found in these codons (9). In a previous study, we demonstrated that mutations in the *β-catenin* gene occured in rat tumors of specific tissues or organ sites and in a carcinogen-specific manner (13). In the present study, we found a total of 5 mutations, all at codons 32 and 34 and all were G:C to A:T transition mutations. These results suggest that carcinogens such as AOM and DSS preferentially affect the N-terminal half (around codon 33) of the GSK3β sites of the *β-catenin* gene. This seems to be a characteristic aspect seen with rat colon tumors induced by a specific carcinogen (18).

Reduced expression levels of the *MGMT* mRNA and protein have been found in human colon carcinoma and this status was associated with aberrant hypermethylation in the promoter region of the *MGMT* gene (19). It was also suggested that inactivation of the *MGMT* gene caused by aberrant promoter methylation induces G:C to A:T transition mutations of the *K-RAS* genes but not of the *β-catenin* gene (7, 20, 21). We found that the CpG islands in the promoter region of the rat *Mgmt* gene were methylated in all 11 tumors examined in the present study by using the COBRA technique (data not shown). Regardless of the DNA methylation status, methylated histone, methyl-CpG binding protein and mutation of the *MGMT* gene cause the reduction in expression level of *MGMT* mRNA in human colon carcinoma cell lines, thus contributing to the impaired function of the MGMT protein (22, 23). These findings suggest that methylation of the promoter region of the *Mgmt* gene may be a minor event in AOM/DSS-induced rat colon carcinogenesis.

β-Catenin gene mutations and the accumulation of the β-catenin protein were found in preneoplastic lesions such as ACF, β-catenin accumulated crypts (BCAC) and mucin-depleted foci (MDF) (9, 24-26). However, the association between *Mgmt* expression and *β-catenin* gene mutation remains unclear in these lesions. This issue warrants further study.

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