

Regular article

Methionine Sulfoxide Stimulates Hepatocarcinogenesis in Non-alcoholic Steatohepatitis (NASH) Mouse: Possible Role of Free Radical-mediated DNA Methylation

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We have reported the formation of 5-methylcytosine from cytosine *in vitro*, with methyl radicals generated from methionine sulfoxide (MetO). To confirm this reaction *in vivo*, MetO was added to the drinking water and administered to non-alcoholic steatohepatitis (NASH) mice, which develop hepatitis caused by endogenous oxidative stress. Histopathological examinations revealed incidences of hepatocellular carcinoma of 16.7% and 90% in the 0% and 3% MetO groups, respectively. Higher DNA methylation was detected in the promoter region of the *p16* gene isolated from the livers of MetO-treated mice. The higher incidence of liver tumors may be due to the methyl radical-mediated formation of 5-methylcytosine in DNA, which triggers epigenetic changes.

Key words: methionine sulfoxide (MetO), DNA methylation, NASH, epigenetic, free radical

Introduction

Many previous studies have revealed the mutational events leading to the activation of oncogenes or the inactivation of tumor suppressor genes in carcinogenesis. There is accumulating evidence that epigenetic changes are a driving force in cancer development, in addition to genetic changes. During multistep hepatocarcinogenesis, CpG island methylation of tumor suppressor genes, such as *APC*, *GST1*, *RASSF1A*, *p16*, *COX-2*, and *CDH1* (*E-cadherin*), occurs frequently and accumulates gradually (1,2). The hypermethylation of CpG islands in the promoter regions of these genes is correlated with the lack of gene transcription. It was suggested that the aberrant DNA methylation associated with alterations in DNA methyltransferase (DNMT) may promote hepatocarcinogenesis (3). However, the DNMT expression level and the DNA methylation status are not always correlated (4,5). Hypermethylation seems to be

closely related with chronic inflammation (6) and reactive oxygen species (ROS) generation (7–10). We have reported the formation of 5-methylcytosine in DNA by free radical mechanisms *in vitro*, using methyl radical generating systems such as cumene hydroperoxide (CuOOH)/Fe (11) and methionine sulfoxide (MetO)/Fenton reagent (H₂O₂, Fe²⁺, ascorbic acid, EDTA) (12). CuOOH is a known tumor promoter in the 7,12-dimethylbenz[*a*]anthracene (DMBA)-initiated two-stage carcinogenesis model in mouse skin (13). It would be interesting to confirm whether DNA methylation and cancer induction are enhanced by the administration of MetO to mice with high oxidative stress due to chronic inflammation, because methyl radical-mediated seeding of DNA methylation may occur at a very early stage of cancer development and trigger DNA hypermethylation, epigenetic changes and carcinogenesis (14). As a prelude to this approach, MetO, an oxidized form of methionine, was administered to non-alcoholic steatohepatitis (NASH) mice with high oxidative stress in the liver due to fibrosis and cirrhosis, induced by a high fat diet (15), and DNA methylation and carcinogenesis were examined.

Materials and Methods

Chemicals: L-Methionine was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were the highest quality commercially available.

Preparation of MetO: L-Methionine (66.6 g) was

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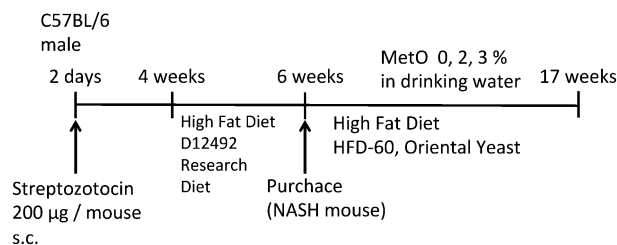


Fig. 1. The experimental protocol of MetO administration to NASH mice.

dissolved in 666 mL of 20 mM phosphate buffer (pH 7.4), and then 50 mL of 30% H_2O_2 was added with stirring. The mixture was stirred for 5 h and allowed to stand for 3 h at room temperature. After the reaction, MetO was recrystallized from ethanol. The final product showed a single peak by HPLC (Column: Capcell Pak C18 MG, 5 µm, 4.6 × 250 mm, Shiseido Fine Chemicals, Tokyo, Japan; elution: methanol: CH_3CN : H_2O = 20:15:65, containing 0.1% TFA).

Animals and experiment: Male STAM MiceTM C57BL/6N-NASH (6 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). This mouse is an animal model of NASH on a basis of streptozotocin induced insulin resistance and high fat diet. This model is produced from C57BL/6N strain without genetic change. Streptozotocin induces oxidative stress in the liver (16). Mice were maintained on a 12-h light/dark cycle and were given free access to a high fat diet (HFD-60, Oriental Yeast Co., Tokyo, Japan) and water. The 26 mice were divided into 3 experimental groups (6 mice for the control and 10 mice for the two MetO groups). The mice in each group were given 0, 2 or 3% MetO in their drinking water for the entire duration of the experiment (Fig. 1). The drinking water was replaced on a daily basis. At week 11 of the experiment (17 weeks old), the mice were sacrificed for macroscopic and histopathological assessments of the livers and immunohistochemical examinations. Liver samples were preserved in formalin until the histopathological and immunohistochemical examinations, and the DNA methylation analysis. Male C57BL/6N mice (Charles River Lab, feedstuff: CE-2, Kyudo Co., Tosu, Japan) were also used as a non-NASH control for the DNA methylation analysis. All of the animal experimental procedures were performed in accordance with the guidelines for the care and use of laboratory animals at our university.

Macroscopic analysis of livers: A surface nodule larger than 1 mm in diameter in the left lobe of the liver was counted as a neoplastic nodule.

Quantitative analysis of histological parameters: HE (hematoxylin and eosin) staining for histopathological classification and assessment of incidence and

multiplicity of hepatocellular adenomas (HCAs) and carcinomas (HCCs) in mice was performed at week 17. Examinations were performed on the basis of the characteristic staining patterns for mouse HCAs and HCCs, according to published criteria (17,18).

Histopathological classification of liver tumors: Macroscopically visible lesions were classified as either hepatocellular adenoma (HCA) or carcinoma (HCC), according to the following criteria: (i) HCAs are considered to be larger than one lobulus, with signs of compression at the border. They were diagnosed as benign lesions if less than three criteria described for hepatocellular carcinomas were applicable. Staining patterns (basophilic/amphophilic, eosinophilic/clear cell and mixed cell phenotypes) were observed in nodules. (ii) For the diagnosis of HCCs, three or more of the following criteria were required: (a) basophilia, (b) undifferentiated trabecular structure, (c) evidence of invasive growth and occasionally of metastasis, (d) nuclei larger than those in the normal-appearing tissue and rich in chromatin, (e) high incidence of mitosis, or appearance of atypical mitotic figures (17).

Immunohistochemical examinations: Cytokeratin (CK) 8/18, and proliferating cell nuclear antigen (PCNA) staining were performed for the samples obtained at week 17, according to the method of Kakehashi *et al.* (19).

Methylation-specific PCR: DNA methylation of the CpG islands of the *p16* promoter region was determined by bisulfite modification of genomic DNA and subsequent methylation-specific PCR. The DNA was extracted with a DNA extraction kit (NucleoSpin Tissue, Macherey-Nagel, GmbH & Co. KG, Düren, Germany) from formalin-fixed liver. Bisulfite modification of DNA (1 µg) was performed with a Methyl EasyTM Xceed kit (Human Genetic Signatures Pty Ltd., North Ryde, Australia). Bisulfite-modified DNA was amplified with a methyl-specific primer set (M forward, CGATTGGGCGGTATTGAATTTTCGC; M reverse, CACGTCATA CACACGACCCTAAACCG) and a non-methyl-specific primer set (U forward, GTGATTGGGTGGGTATTGAATTTTGTG; U reverse, CACACATCATAC ACACAACCCTAAACCA) for the mouse *p16* promoter site (20). The methylated DNA amplification conditions were as follows: initial denaturation at 95°C for 2 min, denaturation at 94°C for 30 sec, annealing 66°C for 1 min, and extension at 72°C. The non-methylated DNA amplification followed the same procedure, except that the annealing temperature was 64°C. Amplification products were separated by gel electrophoresis and stained with ethidium bromide.

Statistics: Statistical analyses were performed with Dunnett's multiple comparison test.

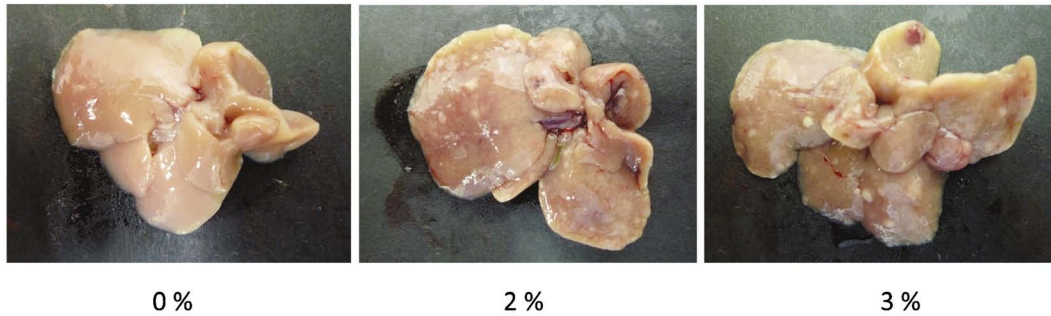


Fig. 2. The neoplastic nodules induced by MetO administration on the surfaces of livers.

Table 1. Incidence and multiplicity of liver tumors

treatment	No. mice	Incidence (%)			Multiplicity (No./mouse)		
		HCA	HCC	Total	HCA	HCC	Total
0%	6	6 (100)	1 (16.7)	6 (100)	3.33 ± 3.01	0.33 ± 0.82	3.67 ± 3.78
2%	10	10 (100)	8 (80.0)**	10 (100)	5.80 ± 3.74	1.30 ± 0.95	7.10 ± 4.36
3%	10	10 (100)	9 (90.0)**	10 (100)	7.20 ± 3.77	1.70 ± 1.42*	8.90 ± 4.01*

Data are mean ± SD, * $p < 0.05$, ** $p < 0.01$, significantly different from 0% control.

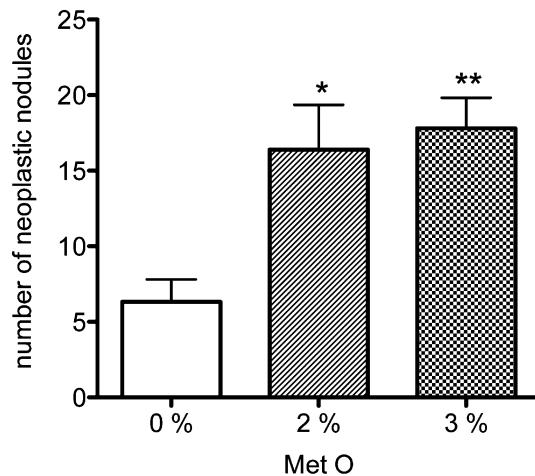


Fig. 3. The numbers of neoplastic nodules induced by MetO administration on the surface of the left hepatic lobe. * $p < 0.05$, ** $p < 0.01$.

Results

The number of neoplastic nodules on the surface of the left hepatic lobe significantly increased with MetO administration (Figs. 2 and 3). Histologically, the incidence of hepatocellular carcinoma (HCC) in NASH mice was significantly increased after the administration of 2 and 3% MetO aqueous solutions (Table 1). The multiplicities of HCC and the total HCCs and HCAs were also elevated by MetO administration (Table 1). Furthermore, the incidences of basophilic and mixed type hepatocellular adenomas (HCAs) were significantly increased in the MetO-treated mice. The multiplicities

of HCAs increased dose-dependently with the concentration of MetO in the drinking water ($p < 0.05$: Spearman's rank correlation coefficient test). As a marker of liver preneoplastic lesions, cytokeratin (CK) 8/18 was employed (19). The number of CK8/18 positive foci was elevated in the MeO-treated animals, but the difference was not significant (data not shown). CK8 and CK18 are known to be distributed in cytoplasmic filament networks and as bands associated with the plasma membrane. Representative pictures of HE, CK8/18 and PCNA staining in basophilic, eosinophilic and mixed type HCCs in serial sections are presented in Fig. 4. Significant overexpression of CK8/18 was detected in the liver preneoplastic lesions and tumors of the MetO-treated mice (Fig. 4). Furthermore, the CK8/18-positive HCCs, HCAs and preneoplastic lesions of the MeO-treated mice were strongly positive for the cell proliferation marker PCNA.

We also analyzed the DNA methylation status in the NASH mice with or without MetO-treatment. We chose the *p16* gene for the DNA methylation analysis because its increased methylation has been observed during hepatocarcinogenesis in rats and most human cancers, including liver cancer. The NASH mice showed higher DNA methylation level of the *p16* promoter in the non-cancerous region, as compared with the control C57BL mice (Table 2). This is the first report of the enhanced DNA methylation of a tumor suppressor gene promoter in the liver of the NASH mouse. The MetO-treated NASH mice had even higher levels of DNA methylation, as compared with the MetO-nontreated NASH mice. Additionally, the methylation of the *p16*

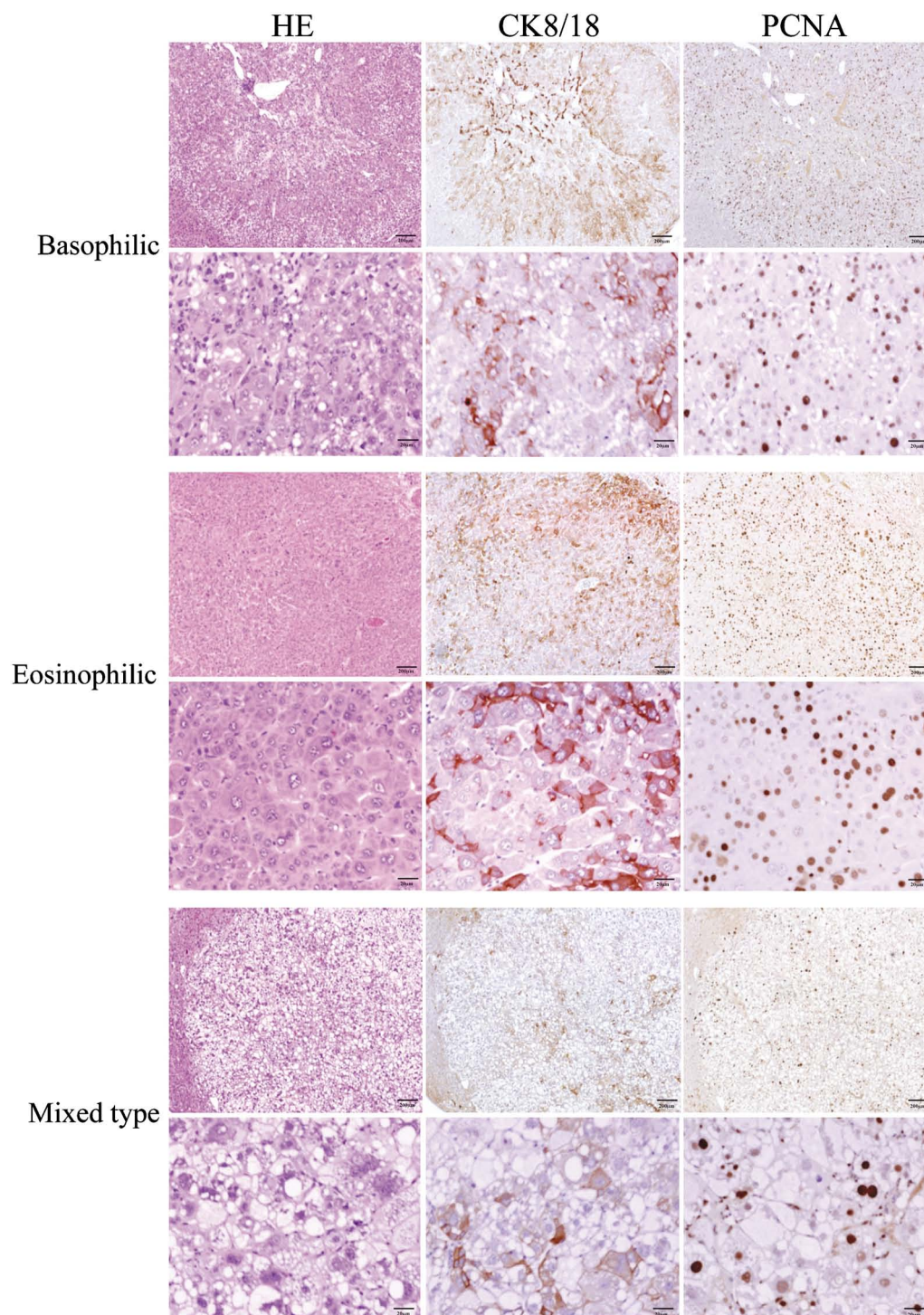


Fig. 4. HE staining and immunohistochemical assessment of CK8/18 and PCNA in the livers of NASH mice treated with MetO.

promoter was clearly detected in the hepatocellular carcinomas induced by MetO administration (Fig. 5).

Discussion

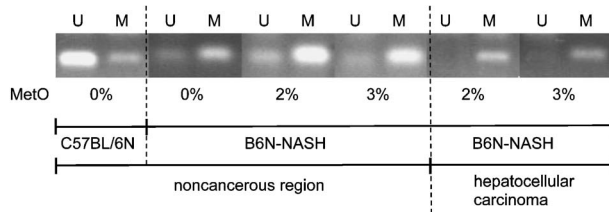
Chronic inflammation is implicated as a cause of epigenetic changes during liver carcinogenesis in humans and animals (6,21). However, its detailed molecu-

lar mechanisms remain unclarified. As endogenous MetO formation in proteins is a potential pathological biomarker related to inflammation (22), aging (23) and smoking (24), it was suggested that MetO is involved in cytosine methylation during epigenetic changes (12,14). Methyl radicals are generated from free and peptide-

Table 2. Methylation status of *p16^{Ink4a}* in MetO-treated mouse liver

Treatment	No. mice	U	U + M	M	M band detected (%)
C57BL	3	3	0	0	0 (0%)
NASH 0%	6	3	1	2	3 (50%)
2%	10	2	6	2	8 (80%)
3%	10	3	3	4	7 (70%)

U: U band was predominant, U + M: both U band and M band were detected, M: M band was predominant.

**Fig. 5.** Methylation of *p16^{Ink4a}* in liver tumor and non-tumor regions of NASH mice after treatment with MetO. The results from the C57BL/6N mouse are a control for those from the NASH mice.

bound methionine sulfoxide (MetO) oxidation by peroxynitrite and hydrogen peroxide/iron(II) *in vitro* (25). Furthermore, we have reported the formation of 5-methylcytosine in DNA by free radical mechanisms *in vitro*, using methyl radical generating systems such as methionine sulfoxide (MetO)/Fenton reagent (12). Our present results suggest that the free radical-mediated cytosine C-5 methylation induced by MetO and reactive oxygen species (ROS) triggers hypermethylation of tumor suppressor genes, such as *p16*, and stimulates liver carcinogenesis. Although our results were obtained using specific mice (NASH) with specialized feeding conditions (MetO), our data may be generalized for humans with high oxidative stress, because both ROS and MetO are formed by oxidative stress. MetO in proteins is repaired to Met by methionine sulfoxide reductase (Msr) in normal cells (26). It is worth mentioning that the Msr gene was identified as a candidate metastasis suppressor in human HCC. The Msr mRNA and protein levels were both decreased in HCC (27). In addition, Msr gene expression is reportedly down-regulated in breast cancer cells (28). Radical cytosine methylation may occur randomly, regardless of the neighboring sequences, and the initial cytosine methylation level may be very low. We think the initial cytosine methylation corresponds to the so-called “seeding methylation”, proposed by Clark and her collaborators (29). A non-random methylation pattern will appear in the subsequent steps. For example, the maintenance DNMT methylates nascent DNA, by recognizing only hemimethylated CpG. Methyl binding domain (MBD) proteins also bind to hemimethylated CpG, in a sequence-specific manner (30), and recruit *de novo* DNMT.

Thus, DNA methylation is spread from the seed (29). Chemical DNA modifications by alkylating agents and free radicals depend partially upon the chromatin structure (31). Such modifications occur with greater frequency at the more accessible open chromatin structures. A similar result was also observed in the cytosine methylation pattern (32). One possible reason why certain CpG islands are hypermethylated in cancer cells, while others remain unmethylated, may be as follows. Some of the methylated CpG in specific sequences may inactivate tumor suppressor genes, thus giving a selective advantage to the survival of pre-cancerous and cancer cells (33). In conclusion, the administration of MetO to NASH mice caused the enhancement of hepatocarcinogenesis and DNA methylation. These results support our hypothesis that the methyl radicals generated from MetO and ROS trigger the hypermethylation of the *p16* promoter and carcinogenesis.

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