Review

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Free radical-mediated cytosine C-5 methylation triggers epigenetic changes during carcinogenesis

Abstract: The methylation of the C-5 position of deoxycytidine (dC) in the promoter regions of tumor suppressor genes is often observed in cancer cells. We found that various environmental agents, as well as endogenous compounds such as methionine sulfoxide (MetO), generate methyl radicals and modify dC to form 5-methyl-dC in DNA *in vitro*. We confirmed that both DNA methylation and cancer incidence in the liver were increased by the administration of MetO to oxidatively stressed mice. In this review, we summarize previous reports on methyl radical generation *in vitro* and *in vivo* and DNA modifications by methyl radicals, including our discoveries, as well as our recent experimental evidence suggesting that free radical-mediated dC methylation triggers epigenetic changes.

Keywords: 5-methyldeoxycytidine; DNA methylation; epigenetic; methyl radical.

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Introduction

DNA methylation at cytosine C-5 in the 5′-CpG-3′ sequence facilitates the organization of mammalian genomes by controlling gene expression in developing embryos (1). CpG-rich regions, termed CpG islands, are present in the 5′ regions of many housekeeping genes and are normally unmethylated (2). During carcinogenesis, hypermethylation of the normally unmethylated tumor suppressor gene promoters frequently occurs, which can lead to gene silencing (3–5). In both *de novo* methylation and maintenance methylation, a methyl group is introduced enzymatically to the C-5 position of cytosine by DNA methyltransferases (DNMTs). One of the widely accepted mechanisms of enhanced *de novo* methylation during carcinogenesis is the overexpression of DNMT3b. For example, the inactivation of tumor suppressor genes during cadmium-induced cell transformation is reportedly due to the induction of DNMT3b before the hypermethylation of these genes (6). *DNMT3b* polymorphisms are also reportedly correlated with prostate and lung cancer risks (7, 8). The *DNMT* genotype with higher activity is related to a more frequent cancer incidence. For example, a C/T polymorphism in the *DNMT3b* promoter region results in increased activity and has been identified as a risk factor for lung cancer (9). However, increased expression of DNMTs is not always correlated with DNA hypermethylation (10). The detailed molecular mechanism underlying the initial methylation in a normal cell before its carcinogenic transformation is still mostly unknown. Inflammation is reportedly involved in DNA hypermethylation and epigenetic changes during carcinogenesis (11). One of the published hypotheses on the mechanism of *de novo* DNA methylation, especially in relation to inflammation, is the HOCl-induced chlorination of DNA at the cytosine C-5 position or the incorporation of the nucleotide product 5-chloro-dCTP into DNA (12). 5-Chloro-dC in DNA mimics 5-methyldeoxycytidine (m^5dC) and is recognized by the maintenance DNMT protein, which triggers further DNA methylation. The same consideration is also possible for another type of inflammation-induced DNA damage, 5-bromo-dC.

We proposed that during carcinogenesis, the normally unmethylated cytosine C-5 in tumor suppressor gene promoters is initially methylated by a free radical

mechanism, for the following reasons: (1) methyl radicals are produced from various environmental agents and endogenous compounds, including methionine sulfoxide (MetO) in the presence of Fe^{2+} or oxygen radicals *in vitro* (for further information, see the next section). (2) Methyl radicals are produced by various well-known carcinogens or tumor promoters, such as dimethylhydrazine (DMH), acetaldehyde, *tertiary*-butyl hydroperoxide (tBuOOH), and cumene hydroperoxide (CuOOH), by their metabolism. (3) DNA methylation inducers, including inflammation (11), aging (13), and smoking (14), as well as many epi-mutagens, such as nickel, chromate, arsenite, hydroxyurea, cisplatin, and diethylstilbestrol, directly or indirectly generate oxygen radicals (15–17) and may trigger the formation of methyl radicals from the endogenous molecule, MetO. (4) The cytosine C-5 position is targeted by free radical reactions, based on quantum chemical calculations (18).

Based on these previous findings, it was tempting to test the reaction of dC with the methyl radical-producing tumor promoters tBuOOH and CuOOH in the presence of Fe^{2+} because they are known to methylate deoxyguanosine (dG) at the C-8 position by a free radical mechanism. The formation of m^5dC was clearly demonstrated in these *in vitro* reactions, although the yield was lower than that of methylated dG, 8-methyldeoxyguanosine (m^8dG) (19) (Figure 1). Oxidized methionine, MetO, also generated methyl radicals in the presence of hydroxyl radical (·OH) and induced the dC→m^5dC modification (20). Increased MetO formation in proteins has been detected in conjunction with inflammation, aging, and smoking (21–24), thus suggesting a link between free radical-mediated dC methylation and epigenetic changes during carcinogenesis (25).

In this review, we summarize the previous reports on the generation of methyl radicals *in vitro* and *in vivo*, the DNA modifications induced by methyl radicals, including our discoveries, and our recent experimental evidence suggesting that free radical-mediated dC methylation triggers epigenetic changes.

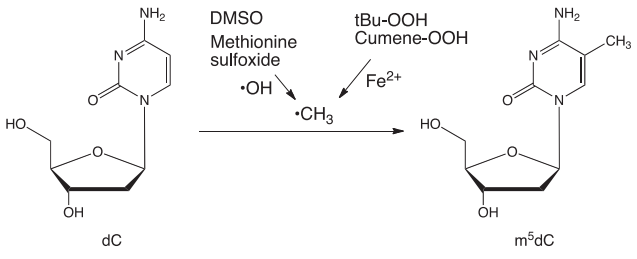


Figure 1 Formation of m^5dC *via* a methyl radical by DMSO and MetO and by tBuOOH and CuOOH.

Generation of methyl radicals and their reactions with nucleic acid components

Various examples of the generation of methyl radicals from endogenous and exogenous compounds and their reactions with nucleic acid components have been reported. In many cases, reactive oxygen species (ROS) have been implicated as essential factors in methyl radical production from chemicals, and iron is often involved in ROS production.

tBuOOH produces methyl radicals by metal catalysis. Maeda et al. (26) first reported the methyl radical-mediated modification of nucleic acid components. Guanine, guanosine, and 5′-guanilic acid were methylated with tBuOOH in the presence of ferrous ion to yield the corresponding 8-methyl derivatives, especially under acidic conditions. Adenine and adenosine also underwent methylation to produce the C2- or C8-methyl derivatives. Hix et al. (27) also reported methyl radical generation from the tBuOOH-Fe^{2+} system and adduct formation, including m^8dG, in DNA. The reaction of a hydroxyl radical with *t*-butyl alcohol, the major metabolite of the carcinogenic gasoline additive methyl *t*-butyl ether, produces the alkoxyl radical, which can undergo spontaneous fission to produce acetone and the methyl radical (28). The human carcinogen acetaldehyde generates a methyl radical upon treatment with peroxynitrite and Fe^{2+}/H_2O_2 (29). Furthermore, Kang et al. (30) reported that methyl radicals were generated from dimethylsulfoxide (DMSO), with hydroxyl radicals produced from a Fenton-type reaction between hydrogen peroxide and iron(II)-EDTA. When RNA was reacted with this system, 8-methylguanine, 2-methyladenine, and 8-methyladenine were detected in the acid hydrolysate. Makino (31) reported methyl radical production in a γ-irradiated aqueous solution of DL-methionine, as detected by ESR spectroscopy. Nakao et al. (32) reported that both free and peptide-bound MetO are oxidized by hydrogen peroxide/iron(II)-EDTA and peroxynitrite, through a hydroxyl radical intermediate, to produce a methyl radical.

Various descriptions of methyl radical formation *in vitro* in relation to AGE (advanced glycation end-product), a methylcobalt compound, and a biochemical cofactor have been published. Nakayama et al. (33) reported that the free radicals generated by methylglyoxal (an AGE compound) and hydrogen peroxide included the methyl radical. Kofod (34) reported that pentaaminemethylcobalt (III) decomposes upon dissolution in water by releasing a

methyl radical, which reacts with AMP and GMP to afford 8-methyl-AMP and 8-methyl-GMP, respectively. Methyl radical generation was suggested during methyl transfer from methylcobalamin, a cofactor involved in biomethylation reactions such as the methylations of homocysteine and heavy metals, to diaquocobinamide (35).

Methyl radicals are also generated by the metabolism of chemicals. In the horseradish peroxidase-catalyzed oxidation of the colon carcinogen 1,2-DMH, the ESR spectra of the 2-methyl-2-nitrosopropane spin-trapped radicals revealed the methyl radical (36). Additionally, the metabolism of DMH by liver microsomes revealed methyl radical generation *in vitro* (37).

Several examples of methyl radical formation *in vivo* or in cultured cells have been published. Nakao et al. (38) performed EPR spin trapping studies and reported that acetaldehyde metabolism by xanthine oxidase, submitochondrial particles, and in whole rats generates methyl radicals. An α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN)-methyl radical adduct was also detected in the bile of acetaldehyde-treated rats. 8-Methylguanine was found in the liver and colon DNA of rats treated with DMH (39). The anticancer hydrazine derivative procarbazine, which also has carcinogenic properties, generates methyl radicals in rat organs (40). When tBuOOH was administered to rats, methyl radical generation was detected by the EPR spin trapping technique, and methyl radical-dG adduct formation was observed in the liver and stomach DNA (41). Methyl radicals were also detected when isolated mouse keratinocytes were treated with the skin tumor promoters, tBuOOH and CuOOH, in the presence of spin traps (42). Evidence for the metabolism of these organic peroxides into methyl radicals by human carcinoma skin keratinocytes was obtained by an ESR spin trapping study (43).

To determine whether a skin tumor promoter could induce radical methylation of DNA *in vivo*, mouse skin was treated with CuOOH in the presence of Fe^{2+}, and the methylated purine nucleosides in the epidermal DNA were analyzed by an LC-MS-MS method (44). 8-Methyldeoxyadenosine (m^8dA) and m^8dG were detected at levels of 2.65±1.41 and 0.98±0.14 per 10^7 nucleotides, respectively, suggesting that radical DNA methylations, including the formation of m^5dC, may occur in cellular DNA. These results are compatible with our hypothesis that *de novo* DNA methylation at cytosine C-5, triggered by a free radical mechanism, may be related to epigenetic changes in carcinogenesis.

Regarding nucleic acid modifications by methyl radicals, only the formation of 8-methylguanine, 2-methyladenine, and 8-methyladenine has been reported. The generation of 5-methylcytosine has not been detected, possibly due to its low yield. *In vivo*, a small increase of 5-methylcytosine in DNA would be especially difficult to detect because it is a normal DNA constituent.

Methylation at the C-5 position of cytosine by a methyl radical

The C-5 position of cytosine can be methylated by environmental chemicals because it is an active site for free radical reactions (18). We found m^5dC as a reaction product of CuOOH and deoxycytidine (dC) under physiological conditions (pH 7.4) in the presence of ferrous ion (19) (Figure 1). m^5dC was also formed by the reaction of tBuOOH and dC under the same conditions. As CuOOH and tBuOOH are both tumor promoters, these non-enzymatic methylation reactions are particularly interesting. These chemicals are widely used in industry. CuOOH is a synthetic intermediate for acetone and phenol production, and tBuOOH is an initiator for radical polymerization. Therefore, humans are exposed to these organic peroxides during manufacturing processes (45). It is worth mentioning that m^5dC formation by CuOOH was strongly inhibited by the free radical scavengers 2,2,6,6-tetramethylpiperidine-1-oxyl and POBN. This finding supports the idea that cytosine methylation occurs *via* methyl radical generation from environmental chemicals, such as CuOOH or tBuOOH. The rate of dC methylation in DNA by CuOOH is about 10 times slower than that of monomeric dC under the same conditions [63 mM CuOOH and 6 mM Fe(II) at pH 7.4] (19). This rate difference is probably due to the steric hindrance of double-stranded DNA, which often occurs in chemical modifications of DNA. For example, when dG, as the monomer or in DNA, was reacted with oxygen radicals, a 16-fold higher yield of 8-hydroxydeoxyguanosine (8-OHdG) from dG was observed, as compared with its formation in DNA (46).

The formation of m^5dC from dC or in DNA *in vitro* was also observed in treatments with DMSO and MetO, under physiological conditions at pH 7.3 in the presence of the Fenton reagent (20). The production levels of m^5dC in the DNA-100 mM DMSO-Fenton and DNA-100 mM MetO-Fenton reactions were $5/10^4$ dC and $1.6/10^4$ dC, respectively. In addition, m^8dG was detected in DNA under the same reaction conditions.

We recently confirmed the increases in DNA methylation and cancer incidence by the administration of MetO to mice (47). In that study, instead of the Fenton reaction *in vitro*, non-alcoholic steatohepatitis (NASH) mice were used for higher ROS production. NASH mice develop hepatitis

caused by endogenous oxidative stress. The incidences of hepatocellular carcinoma were 16.7%, 80%, and 90% in the groups provided drinking water containing 0%, 2%, and 3% MetO, respectively (Figure 2). The DNA methylation status of the *p16* gene promoter region was higher in the liver of the MetO-treated mice (in both the non-tumor region and the liver tumor) (Figure 3). The higher incidence of hepatocarcinoma may be due in part to the m^5dC formation by methyl radicals *in vivo*. In addition, we confirmed the formation of m^8dA in the MetO-treated mouse liver DNA, suggesting that methyl radicals are actually produced in the mouse liver by those treatments (44). Notably, MetO is generated in proteins by inflammation, aging, and smoking (21–24), which are recognized as DNA hypermethylation inducers (11, 13, 14). In addition, MetO reductase (*Msr*), which repairs MetO in proteins, is reportedly a tumor suppressor (48).

Discussion

The hypermethylation of the tumor suppressor gene promoter DNA is considered to play an important role in carcinogenesis. Clark and her collaborators (49) suggested that the hypermethylation of the *GSPT1* promoter is initiated by a combination of transcriptional gene silencing and methylation seeds. However, the precise mechanism of the initial seeding methylation is not known. It was also reported that *de novo* (initial) methylation occurs in nucleosome-free linker regions, which can be accessed by DNMTs (50). Ushijima and his collaborators (51) reported that RNA polymerase II (pol II) binding around a transcription start site can function as a protective factor for DNA methylation. They speculated that pol II forms a complex with transcription factors, and its binding around promoter CpG islands may compete with DNMTs. If we consider the free radical mechanism for seeding methylation, instead of enzymatic methylation, then site-specific methylation may also depend on the open or closed structure of DNA, due to protein binding. For example, we observed that 8-OHdG, a form of oxidative DNA damage induced by an OH radical, was not formed and distributed uniformly throughout the whole genome but was restricted to particular genes and regions, depending upon the chromatin structure (52).

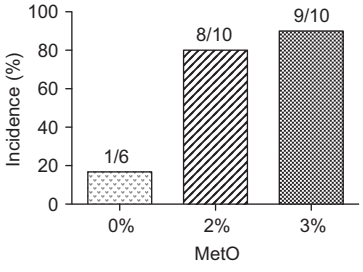


Figure 2 Incidence of hepatocellular carcinoma after administration of MetO to NASH mice.
The numbers above the bar represent the numbers of tumor bearing mice/total number of mice.

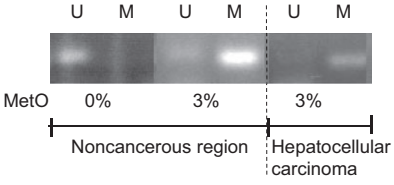
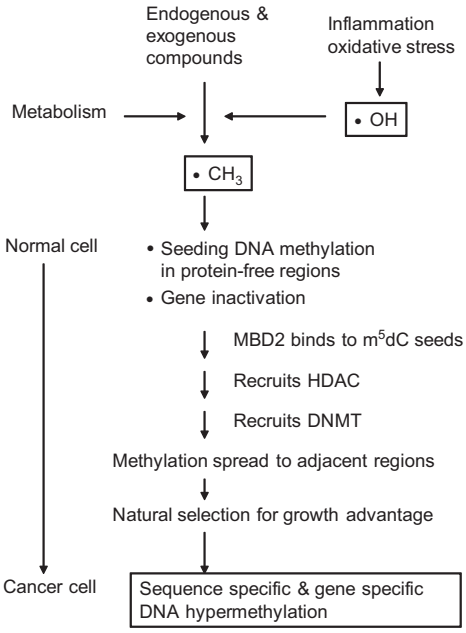


Figure 3 Methylation of *p16* in liver tumor and non-tumor regions of NASH mice after treatment with MetO.

Oxidative stress seems to play an important role in epigenetic changes during carcinogenesis (53–55). Considering the mitochondrial localization of high concentrations of oxygen radicals and MetO (56), the mitochondria (mt) might be a potential source of methyl radical generation, involved in cytosine C-5 methylation. In fact, enhanced cytosine methylation of liver mtDNA in selected regions of the mt genome, such as the mitochondrially encoded NADH dehydrogenase 6 gene, and its decreased expression have been implicated in the pathogenesis of NASH (57). DNMT1 is also reportedly present in the mitochondrial matrix, where it methylates mtDNA, suggesting the presence of epigenetic modifications of the mitochondrial genome in the regulation of mitochondrial transcription (58). In addition, insulin resistance in non-alcoholic fatty liver disease is epigenetically regulated by the methylation of the peroxisome proliferation-activated receptor γ coactivator 1α promoter and is accompanied by mitochondrial dysfunction (59).

Bernal et al. (60) recently reported that a low-dose ionizing radiation (0.7–7.6 cGy) exposure during gestation significantly increased DNA methylation at viable yellow agouti (A^{vy}) locus in male offspring. Their coat color, which is epigenetically controlled, was concomitantly shifted from yellow to pseudoagouti. Maternal dietary antioxidant supplementation mitigated both the DNA methylation changes and coat color shift. These results suggested that ionizing radiation elicits epigenetic changes mediated by oxidative stress. It is reasonable to speculate that the initial seeds of methylation are formed by a free radical mechanism because methyl radicals are generated by the γ-irradiation of methionine, an endogenous molecule (31).

Our hypothesis for free radical-mediated m^5dC formation, in conjunction with a model of DNA hypermethylation during carcinogenesis by Stirzaker et al. (49), is shown in Scheme 1. Even if the level of free radical-mediated m^5dC



Scheme 1 Free radical-mediated m^5dC formation and DNA hypermethylation during carcinogenesis.

formation is very low, it will gradually increase because a specific repair system for m^5dC has not been found. Methyl radicals are presumably able to modify cytosine in any of the CpG, CpA, CpC, and CpT sequences. However, only methylated CpG is recognized by DNMT1, and the methylation patterns can be maintained from the parental to the newly synthesized daughter DNA strands in DNA replication, whereas m^5dC in other sequences would disappear after DNA replication. In addition, only methylated CpG in a specific sequence context can be recognized by methyl-CpG binding domain proteins (MBD2 and MeCP2), which recruit DNMT3 and histone deacetylase (61). These proteins promote *de novo* methylation spreading and deacetylation, respectively. Finally, dense DNA methylation and stronger gene silencing may occur. Therefore, an increase in non-specific cytosine C-5 methylation seeds may enhance the probability of functionally important methylation changes. Natural selection, instead of molecular targeting, is also involved in the formation of gene- and tissue-specific methylation patterns in cancer cells (62). Certain CpG islands are hypermethylated in cancer cells, whereas others are not because the hypermethylation confers a selective advantage to the survival or growth of the cancer cells.

In addition to the DNA hypermethylation of tumor suppressor gene promoters, global hypomethylation is also often observed in cancer cell DNA. Under oxidative stress conditions, oxygen radicals result in the oxidation of dG to 8-OHdG and m^5dC to 5-hydroxymethyldeoxycytidine (5-hmdC) in DNA. The formation of either 8-OHdG or 5-hmdC in the m^5CpG sequence inhibits the DNMT1-mediated methylation of cytosine in the opposite DNA strand, thus generating global hypomethylation (12, 63). Therefore, both the promoter hypermethylation and the global hypomethylation can be explained by a free radical mechanism. Our studies suggested that free radical-mediated cytosine C-5 methylation is a possible mechanism of seeding methylation. Our experimental data revealed that oxygen radicals exert epigenetic as well as mutagenic changes. It should be emphasized that there are no contradictions between our hypothesis and the presently accepted concepts of cancer epigenetics.

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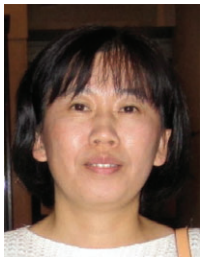

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