Disturbance of DNA methylation patterns in the early phase of hepatocarcinogenesis induced by a choline-deficient L-amino acid-defined diet in rats

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The authors investigated the DNA methylation patterns of the *E-cadherin*, *Connexin 26* (*Cx26*), *Rassf1a* and *c-fos* genes in the early phase of rat hepatocarcinogenesis induced by a choline-deficient L-amino acid-defined (CDAA) diet. Six-week-old F344 male rats were continuously fed with the CDAA diet, and three animals were then killed at each of 4 and 8 days and 3 weeks. Genomic DNA was extracted from livers for assessment of methylation status in the 5′ upstream regions of *E-cadherin*, *Cx26*, *Rassf1a* and *c-fos* genes by bisulfite sequencing, compared with normal livers. The livers of rats fed the CDAA diet for 4 and 8 days and 3 weeks were methylated in *E-cadherin*, *Cx26* and *Rassf1a* genes, while normal livers were all unmethylated. In contrast, normal livers were highly methylated in *c-fos* gene. Although the livers at 4 days were weakly methylated, those at 8 days and 3 weeks were markedly unmethylated. Methylation patterns of CpG sites in *E-cadherin*, *Cx26* and *Rassf1a* were sparse and the methylation was not associated with gene repression. These results indicate that gene-specific DNA methylation patterns were found in livers of rats after short-term feeding of the CDAA diet, suggesting gene-specific hypermethylation might be involved in the early phase of rat hepatocarcinogenesis induced by the CDAA diet. (*Cancer Sci* 2007; 98: 1318–1322)

It is well known that unequivocal liver tumors can be induced by prolonged feeding of rats with a CD diet.[1–3] The choline deficiency causes fatty liver, cirrhosis and HCC in rats.[1–3] Possible mechanisms underlying liver carcinogenesis from the CD diet have been proposed to include the following: liver necrosis associated with subsequent regeneration;[4,5] induction of oxidative DNA damage and lipid peroxidation;[6–9] and generation of genetic alterations.[10,11] It has also been considered that DNA hypomethylation might play an important role in liver carcinogenesis induced by methyl donor deficiency.[12,13] So far, hypomethylation of the *c-fos*, *c-myc*, and *c*-Ha-*ras* genes has been detected in the livers of rats fed with the CD diet.[14,15]

The CDAA diet used in the present study is semi-synthetic, and provides stronger carcinogenic effects than the CD diet in rats.[16,17] The authors have previously reported the hypomethylation of *c-myc* in HCC resulting from the CDAA diet in rats.[18] In another study, hypermethylation of the *E-cadherin* and *Cx26* genes was also detected in those tumors.[19] While genome-wide hypomethylation occurs in several human cancer cells, site-specific hypermethylation such as CpG islands of tumor suppressor genes, is also found.[20] It has been suggested that aberrant DNA methylation of promoter regions of genes is the major mechanism of gene silencing in the development of tumors.[21,22] In fact, aberrant DNA methylation has been found in a variety of human cancers, including liver tumors.[23–26] However, it is unclear why DNA hypermethylation occurs in rat HCC induced by the CDAA diet despite methyl donor deficiency. Therefore, to better understand the disturbance of DNA methylation under methyl donor deficiency, the authors investigated DNA methylation status in the *E-cadherin*, *Cx26*, *Rassf1a* and *c-fos* genes, and measured expression levels of the *Dnmt1* gene in livers with short-term feeding of the CDAA diet in rats.

Materials and Methods

Animals and treatment. A total of 12 F344 male rats, 5 weeks old, were purchased from Japan SLC Inc. (Shizuoka, Japan), and were housed three per plastic cage containing white flake bedding, in an air-conditioned room, at a constant temperature of 25°C, and a 12-h light–dark cycle. Food and water were available *ad libitum* throughout the study. After a 1-week acclimation period on basal diet in pellet form (CF-2 Diet; Clea Japan, Tokyo, Japan), nine animals received the CDAA diet (product number 518753; Dyets Inc., Bethlehem, PA, USA), consisting of ingredients as previously described.[16,17] Subgroups of three rats were killed by exsanguination from the abdominal aorta, under light ether anesthesia, at 4 and 8 days and 3 weeks after the beginning of the experiment. To obtain normal liver tissues, three rats were also killed at 6 weeks of age without the CDAA diet feeding.

Tissue preparation. Upon killing, whole livers were immediately excised and frozen in liquid nitrogen, and stored at –80°C until analysis. Part of the livers was fixed in 10% neutral buffered formalin at 4°C, routinely processed for HE staining, and histopathologically evaluated according to diagnostic criteria as previously described.[16,17]

Bisulfite sequencing. Bisulfite treatment of genomic DNA was performed as previously described.[19,27] Briefly, genomic DNA was extracted from pooled liver samples of three rats in each subgroup, using a DNeasy tissue kit (Qiagen, Hilden, Germany), and 500 ng of each sample was denatured in 0.3 M NaOH. Then, 2.9 M sodium bisulfite (Sigma, St Louis, MO, USA) and 0.5 mM hydroquinone (Sigma) were added, and the mixture underwent 15 cycles of 30-s denaturation at 95°C, and 15-min incubation at 50°C. Samples were then desalted with a Wizard DNA cleanup system (Promega, Madison, WI, USA), and desulfonated by treatment with 0.3 M NaOH at room

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Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; CD diet, choline-deficient diet; CDAA diet, choline-deficient L-amino acid defined diet; *Cx26*, *Connexin 26*; Dnmt, DNA methyltransferase; HCC, hepatocellular carcinoma; PCR, polymerase chain reaction; RT, reverse transcription.

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Table 1. The primer sequence used in the present study

Gene	Primer sequence	Annealing temperature (°C)
Bisulfite sequencing		
E-cadherin	F: 5′-GGAATAAGGAAGTAAGGAAGTT-3′	56
	R: 5′-CCACATACCTACAACAAAAACA-3′	
Connexin 26	F: 5′-GGAGTGATTTAGGTTTTAGGAGAG-3′	62
	R: 5′-TCCCCACAAATCCTAATAAAAACTAC-3′	
Rassf1a	F: 5′-GGATTAGGTTATAGTATTAGTAAATTAG-3′	62
	R: 5′-TCATAATTCAATAAATTCTAACTCC-3′	
c-fos	F: 5′-TATTTATAGGTGAAAGTTATAGATTG-3′	54
	R: 5′-CACTAATAAAAACTACAAAACAAAACT-3′	
Reverse transcription–polymerase chain reaction		
E-cadherin	F: 5′-CTCCCTGAGCTCGCTGAAC-3′	65
	R: 5′-GTGCCACACAGGAACGACTC-3′	
Connexin 26	F: 5′-ACGTTGGCCTTTTGGTTATG-3′	63
	R: 5′-TGTTGCGGGCTGTACTCAG-3′	
Rassf1a	F: 5′-GCTTCATCAAGGTTCAGCTGA-3′	64
	R: 5′-TCAAAGAGTGCAAACTTGCG-3′	
c-fos	F: 5′-TTGCGCAGATCTGTCCGTCT-3′	65
	R: 5′-GTTGATCTGTCTCCGCTTGG-3′	
Dnmt1	F: 5′-AGAAAGCCAACGGTTGTCCT-3′	64
	R: 5′-GTCTCACTGTCCGACTTGCTC-3′	

temperature for 5 min. After ethanol precipitation with ammonium acetate, DNA was dissolved in distilled water.

For bisulfite sequencing, PCR was performed with the primer sets of rat *E-cadherin* and *Rassf1a* genes as described previously.[19,27] The primer sets for rat *Cx26* and *c-fos* genes were also designed for rat *c-fos* gene (NCBI accession number NW_047454 and GenBank accession number AF126534, respectively). All primer sets are listed in Table 1. The PCR amplification was performed as described previously.[19,27] PCR products were subcloned using a TOPO TA cloning kit (Invitrogen Corporation), and were sequenced with a BigDye terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems Japan Ltd) and an ABI PRISM 310 genetic analyzer (Applied Biosystems Japan Ltd). For each sample, 10 clones were sequenced.

Semi-quantitative RT-PCR amplification for expression of *E-cadherin, Cx26, Rassf1a, c-fos* and *Dnmt1*. Total RNA was extracted from pooled liver samples of three rats in each subgroup, using ISOGEN (Nippon Gene, Inc., Toyama, Japan) and first-strand cDNA was synthesized from 0.2-μg samples with Ready-To-Go Your-Prime First-Strand Beads (Pharmacia Co. Ltd, Tokyo, Japan). To eliminate possible false-positives caused by residual genomic DNA, all samples were treated with DNase.

Semi-quantitative RT-PCR analysis was performed with the primer sets of rat *E-cadherin*, *Cx26*, and *Rassf1a* genes as described previously.[19,27] Primer pairs for rat *c-fos* and *Dnmt1* genes were also designed against rat *c-fos* and *Dnmt1* sequences (GenBank accession numbers X06769 and AF116344, respectively; Table 1). The rat *Gapdh* gene was used as an internal control gene.[27] PCR amplification was carried out in a reaction volume of 20 μL containing 1 μM of each gene primer, 200 μM of each dNTP, 1 × PCR buffer (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA), 0.5 U of AmpliTaq Gold (Perkin Elmer), and 0.5 μL of synthesized cDNA mixture. For each gene, multiple cycles of PCR amplification were tested. The cycle at which a sample having the highest expression reached an amplification plateau was determined, and a cycle number smaller than this was adopted for the analysis. The amplified products were then separated on 2% agarose gels containing 0.05 μg/mL ethidium bromide.

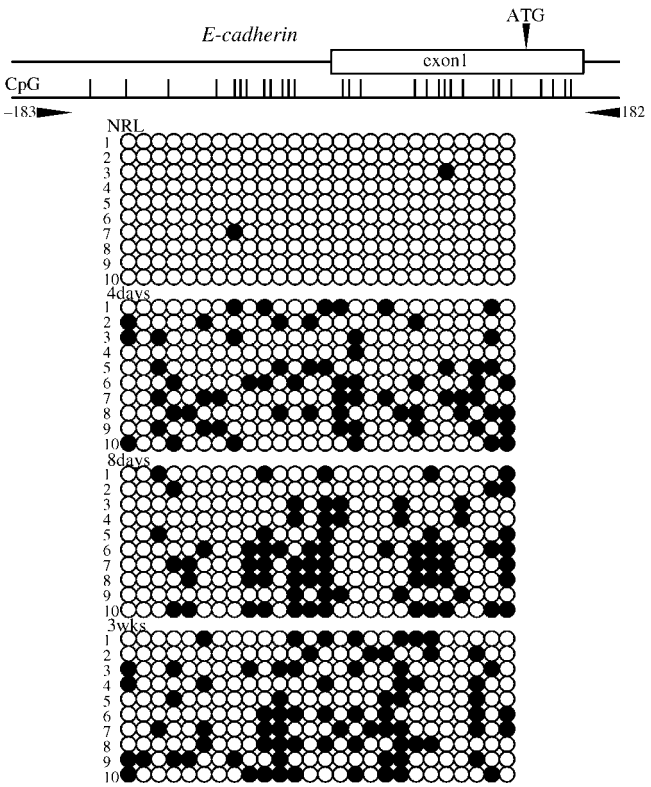

Fig. 1. Methylation analysis of the 5′ upstream region of the *E-cadherin* gene in rat by bisulfite sequencing. The primer pair used for bisulfite sequencing is shown. The transcription start site was defined as +1. Methylated CpG sites are represented by closed circles and unmethylated CpG sites are represented by open circles. NRL, normal liver tissue.

Results

Histologically, in the livers of rats fed the CDAA diet, fat deposits occurred in hepatocytes at 4 days and diffuse fatty

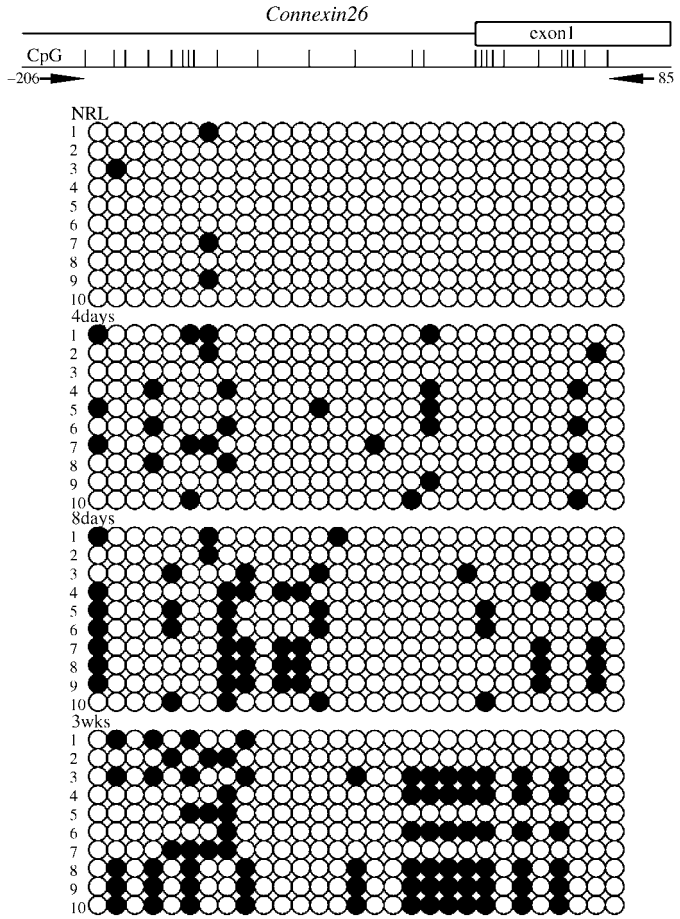

Fig. 2. Methylation analysis of the 5′ upstream region of the *Cx26* gene in rat by bisulfite sequencing. The primer pair used for bisulfite sequencing is shown. The transcription start site was defined as +1. Methylated CpG sites are represented by closed circles and unmethylated CpG sites are represented by open circles. NRL, normal liver tissue.

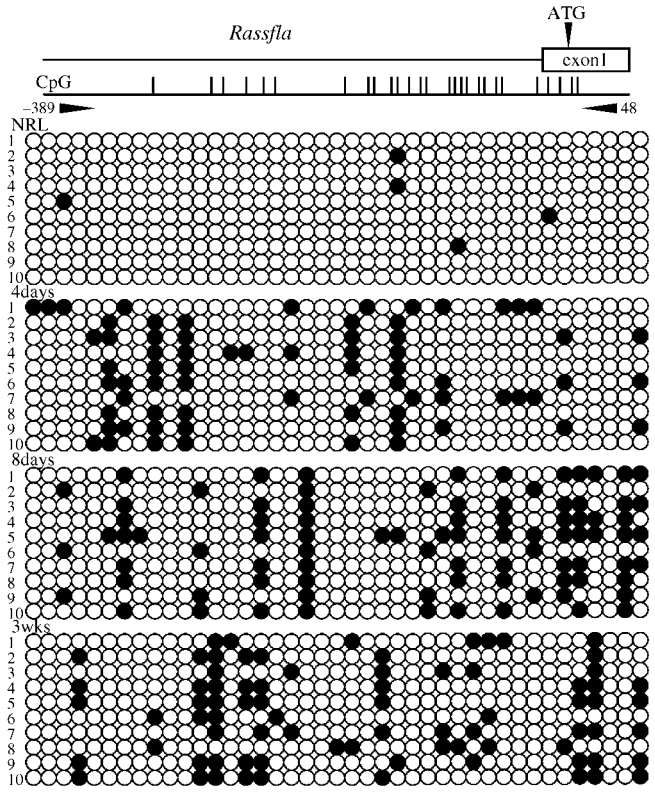

Fig. 3. Methylation analysis of the 5′ upstream region of the *Rassf1a* gene in rat by bisulfite sequencing. The primer pair used for bisulfite sequencing is shown. The transcription start site was defined as +1. Methylated CpG sites are represented by closed circles and unmethylated CpG sites are represented by open circles. NRL, normal liver tissue.

changes were observed at 8 days. Fatty change widely expanded in all areas of livers at 3 weeks and the extension of collagen fibers was observed from Glisson's sheaths.

Results of the bisulfite sequencing analysis for the *E-cadherin*, *Cx26* and *Rassf1a* genes are shown in Figs 1–3, respectively. Methylation status was measured in the 5′ upstream regions of *E-cadherin* (between nt –183 and 182), containing 26 CpG sites; of *Cx26* (between nt –206 and 85), containing 29 CpG sites; and of *Rassf1a* (between nt –389 and 48), containing 28 CpG sites. The livers of rats fed the CDAA diet for 4 and 8 days and 3 weeks were methylated in *E-cadherin*, *Cx26* and *Rassf1a* genes, while normal livers were all unmethylated. The methylation status in the 5′ upstream region of *c-fos* (between nt –202 and 90), containing 19 CpG sites, is shown in Fig. 4. Normal livers were methylated in *c-fos* gene. Although the livers at 4 days were weakly methylated, those at 8 days and 3 weeks were gradually unmethylated.

Expression levels of the *E-cadherin*, *Cx26*, *Rassf1a*, *c-fos* and *Dnmt1* genes in the livers of rats fed the CDAA diet were measured using semiquantitative RT-PCR analysis. Representative results are shown in Fig. 5. No changes in the expression level of *E-cadherin*, *Cx26* and *Rassf1a* were found in the livers of rats fed the CDAA diet. By contrast, while *c-fos* and *Dnmt1* genes weakly expressed in normal livers, these expressions were elevated after 8 or 4 days feeding of the CDAA diet, respectively.

Discussion

The present study indicated that gene-specific changes of DNA methylation patterns occurred in livers of rats after short-term feeding of the CDAA diet, suggesting that gene-specific hypermethylation might be involved in the early phase of rat hepatocarcinogenesis induced by the CDAA diet.

DNA hypomethylation is well known as one of the mechanisms underlying rat hepatocarcinogenesis resulting from a CD diet, because of a multiple methyl group donor-deficiency.[12,13] Indeed, genome-wide hypomethylation of liver DNA has been demonstrated during hepatocarcinogenesis induced by the CD diet.[28,29] Regional hypomethylation of growth-related genes such as *c-fos*, *c-myc*, and *c*-Ha-*ras* has also been detected in the livers of rats after short-term feeding with the CD diet.[14,15] Moreover, hypomethylation of the *c-myc* gene in rat HCC induced by the CDAA diet has been reported.[18] In contrast, site-specific hypermethylation of the *p16* gene in pre-neoplastic lesions and tumors has been reported to occur from folate/methyl deficiency in rat livers.[30] Recently, the authors showed regional methylation in the 5′ upstream regions of the *E-cadherin* and *Cx26* genes in rat HCC as a result of the CDAA diet,[19] and another group reported hypermethylation of intron 1 of neutral endopeptidase 24.11 in rat HCC induced by the same model.[31] Despite methyl-donor deficiency, it is unclear why site-specific hypermethylation occurs in rat hepatocarcinogenesis as a result of the CDAA diet. Although the elevated expression of the *c-fos*, *c-myc* and *c*-Ha-*ras* genes was returned to control levels after ending the methyl-deficient diet feeding, hypomethylation of specific sites in these genes was irreversible.[14,15] In the present

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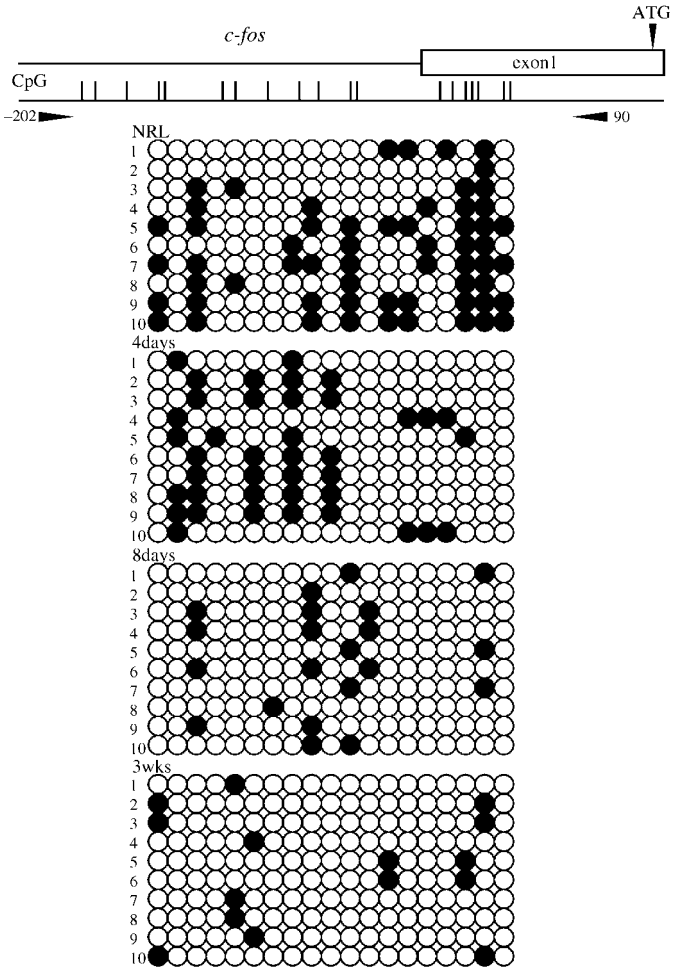

Fig. 4. Methylation analysis of the 5′ upstream region of the *c-fos* gene in rat by bisulfite sequencing. The primer pair used for bisulfite sequencing is shown. The transcription start site was defined as +1. Methylated CpG sites are represented by closed circles and unmethylated CpG sites are represented by open circles. NRL, normal liver tissue.

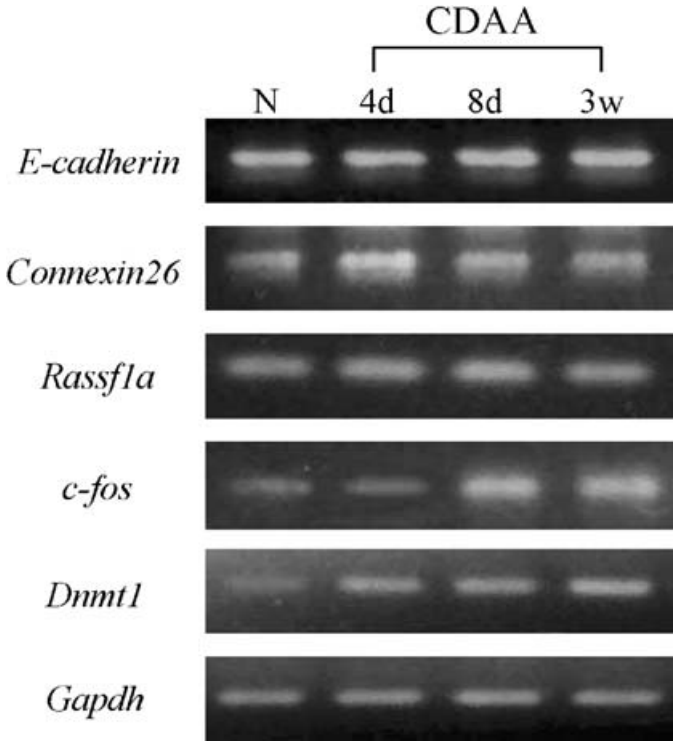

Fig. 5. Expression levels of *E-cadherin*, *Cx26*, *Rassf1*a, *c-fos* and *Dnmt1* mRNAs relative to *Gapdh* mRNA using semiquantitative reverse transcription–polymerase chain reaction analysis. N, normal liver tissue.

study, methylation of CpG sites within the CpG island observed was sparse, not dense, and the methylation was not associated with gene repression. Therefore, gene-specific hyper- and hypomethylation changes might persist and accelerate until the development of HCC under the feeding of the CDAA diet in rats.

Increased expression levels of *Dnmt1* were detected in livers of rats fed with a folate/methyl-deficient diet as well as in HCC.[29] In the present study, the authors also observed elevated *Dnmt1* expression in the livers of rats fed the CDAA diet. Dnmt1 can bind with high affinity to DNA-damaged lesions such as strand breaks, gaps and abasic sites, induced by folate/methyl-deficiency.[29] It is suggested that DNA methylation efficiency due to sequestration from the replication fork might be relative low in DNA containing damaged lesions, resulting in promotion of passive replication-dependent demethylation.[29] In promoter regions of tumor suppressor genes, inappropriate binding of *Dnmt1* may also induce local histone deacetylation, methylation, and regional hypermethylation.[29] In addition, a common oxygen radical-induced guanine derivative, 8-OHdG, could inhibit DNA methylation, resulting in reduced gene expression.[32] 8-OHdG is significantly detectable after only 1 day and progressively accumulates for at least up to 12 weeks in the livers of rats fed the CDAA diet.[16,33] Therefore, it is possible that the aberrant DNA methylation pattern might be due to inappropriate binding of *Dnmt1* to DNA containing damaged lesions, or the accumulation of 8-OHdG in livers of rats fed the CDAA diet.

In conclusion, the present investigation demonstrated that gene-specific methylation changes were found in livers of rats fed the CDAA diet. While global hypomethylation generally occurs in several human cancer cells, CpG islands of tumor suppressor genes are regionally hypermethylated.[20] Despite methyl group donor-deficiency, gene-specific hypermethylation occurs during rat hepatocarcinogenesis induced by the CDAA diet.

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