Hypomethylation of CpG Sites and c-myc Gene Overexpression in Hepatocellular Carcinomas, but Not Hyperplastic Nodules, Induced by a Choline-deficient L-Amino Acid-defined Diet in Rats

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We have investigated aberrant methylation of CpG nucleotides (CpG sites) and gene expression of c-myc during hepatocarcinogenesis induced by a choline-deficient, L-amino acid-defined (CDAA) diet in rats. Male Fischer 344 rats, 6 weeks old, were continuously given a CDAA diet for 50 and 75 weeks and then killed. Macroscopically detectable nodules, which were histologically confirmed to be hyperplastic nodules (HNs) or well-differentiated hepatocellular carcinomas (HCCs), were dissected free from the surrounding tissue. Normal control liver was obtained from 6-week-old rats. Methylation of CpG sites of the c-myc gene was investigated in bisulfite-treated DNA isolated from normal liver, HNs and HCCs. All 33 cytosines in the 5′-upstream region of the c-myc gene were fully methylated in control liver and the 4 HNs. In contrast, these cytosines were completely unmethylated in 5 HCCs. Examination of the c-myc expression by reverse transcription-polymerase chain reaction (RT-PCR) analysis also showed a marked increase as compared to the low levels in normal livers and HNs. These results suggest that hypomethylation of the c-myc gene might play a critical role in malignant transformation from HN to HCC during CDAA diet-induced hepatocarcinogenesis in rats.

Key words: Choline-deficient L-amino acid-defined diet — Hepatocarcinogenesis — c-myc — DNA methylation — Rat

Recently, the physiological roles of DNA methylation and the genetic and epigenetic consequences of aberrations in carcinogenesis have been highlighted. Developmental regulation of gene expression, parental imprinting, inactivation of repeated and foreign DNA and generation of genetic and epigenetic diversity have been described as being normal functions of DNA methylation. Cancer is believed to be due to an accumulation of DNA aberrations, and analysis of DNA isolated from tumors has identified epigenetic and genetic alterations occurring at the target CpG of the (cytosine-5)-DNA methyltransferase (MTase) as one of the most frequent and consistent changes observed in tumor cells. However, the critical role of site-specific DNA aberrations during carcinogenesis has yet to be elucidated.

We have recently established a model for rat liver carcinogenesis induced by chronic feeding of a choline-deficient, L-amino acid-defined (CDAA) diet. This model has advantages for investigating the mechanisms underlying hepatocarcinogenesis due to endogenous factors. The CDAA diet induces 100% hepatocellular carcinomas (HCCs) with frank cirrhosis and a histogenesis through enzymatically altered hepatocyte foci and hyperplastic nodules (HNs). We have demonstrated that continuous cell death and renewal, generation of 8-hydroxydeoxyguanosine (8-OHdG) and 2-thiobarbituric acid-reacting substances (TBARS), and overexpression of c-myc and c-Ha-ras occur during the early stages of carcinogenesis in this model. A critical event is the conversion of HNs, putative preneoplastic lesions, to HCCs. We have so far found that telomerase is activated in both HNs and HCCs induced by the CDAA diet and that mutations of Ki-ras are infrequent in HCCs, with no p53 mutations being detected. Thus, other critical factors must exist for HCC development from HN.

In the present experiment, we studied the methylation status of the c-myc oncogene during CDAA diet-induced hepatocarcinogenesis in rats, since site-specific hypomethylation of c-myc has been reported in human cancer cells and HCCs. Male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka), 6 weeks old at the commencement, were given the CDAA diet (Product number 518753; Dyets Inc., Bethlehem, PA), with the composition described previously. Subgroups of 3 to 5 rats were killed at 50 or 75 weeks old.
weeks after the beginning of the experiment, and 3 rats aged 6 weeks were similarly killed under ether anesthesia to obtain normal control livers. Livers were immediately removed in their entirety, and unequivocal nodules and cancers of rats killed after 50 and 75 weeks were separated from non-tumorous tissues and frozen and stored at −80°C. Portions of each liver sample were also fixed in 10% formalin and routinely processed and stained with hematoxylin and eosin (H&E) for histological examination.

The methylation status of CCGG sites in the 5′ upstream region of the c-myc gene was examined using digested template DNA with a methylation-sensitive endonuclease. Ten nanogram aliquots of genomic DNA from each sample were digested for 16 h with sufficient amounts of MspI or HpaII. After polymerase chain reaction (PCR) amplification, the reaction mixtures were electrophoresed through a 1.5% agarose gel and visualized under UV light.

To investigate the methylation status in detail, the bisulfite-modification method was performed by the procedure described previously, with some modifications. Briefly, 2 µg aliquots of genomic DNA, isolated from frozen tissue by standard phenol extraction, were digested with EcoRI. Ten nanograms of EcoRI-digested genomic DNA suspended in 20 µl of water was denatured by incubation at 94°C for 15 min. The resulting single-stranded DNA was desalted and deaminated by incubation with hydroquinone at a final concentration of 0.3 M NaOH at 37°C for 15 min. Solutions were neutralized by addition of NH₂OAc, pH 7.0, to 3 M, precipitated with ethanol and resuspended in water. The bisulfite-modified DNA was PCR-amplified using primers 5′-AAAAAGGAAGGGGGAGGAT-3′ (sense) and 5′-ACACAAATTTCCCTTCCCTC-3′ (antisense), corresponding to a part of the promoter region and a part of exon 1, respectively. PCR amplification was performed under the following reaction conditions; denaturation step for 5 min at 95°C, and 35 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C. The PCR products obtained were cloned using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and recombinant plasmid DNA clones were sequenced by Sequencing Pro (Toyobo, Tokyo). In each experiment, 5 to 10 clones from different bacterial colonies were investigated. The 5′ upstream region of c-myc gene, which was examined in this study, is shown in Fig. 1.

For semi-quantitative reverse transcription (RT)-PCR analysis, first-strand cDNA was synthesized from 2 µg of total RNA with Ready-To-Go You-Prime First-Strand Beads (Pharmacia, Tokyo). To eliminate the possibility of false positives caused by residual genomic DNA, all samples were treated with DNase. Amplification products comprising a portion of exon 2 for c-myc, and exons 5 through 8 of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) were generated from the cDNA template in parallel PCRs. Primer pairs were as follows: for c-myc, 5′-GAGACTAGGACCTCGAGTACGACT-3′ (sense) and 5′-GGCGCGGGCGAGAGCCGC-3′ (antisense); and for GAPDH, 5′-GGTCTGTAGATTGTCGTGA-3′ (sense) and 5′-GCAATCCGGAATGCTTC-3′ (antisense). PCR amplifications were performed in 20 µl reaction volumes at different concentrations of template cDNA (0.05, 0.5 and 2 µl) for 30 cycles, each consisting of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, using both the c-myc and the GAPDH primers. Each RT-PCR assay was repeated at least once for confirmation.

![Fig. 1. The 5′ upstream region of the rat c-myc gene and the methylation status of CpG sites. The CpG residues that are methylated or unmethylated are indicated in each case based on the results obtained in Fig. 3. \(\text{CpG site; CCGG, HpaII site; NRL, normal liver; HN, hyperplastic nodule; }\) methylated cytosine; ○, unmethylated cytosine.](image)

![Fig. 2. Analysis of the methylation status of the 5′-upstream region of the c-myc gene. Amplification of the 5′-upstream region of the c-myc gene in a normal liver (N), a hyperplastic nodule (HN) and HCCs after digestion with the indicated restriction endonucleases. M, digested with MspI; H, digested with HpaII.](image)
Histological diagnosis of HNs and HCCs was made based on the criteria described previously. Four lesions observed at 50 weeks were HNs and 5 lesions at 75 weeks after the beginning of the experiment were well-differentiated HCCs.

Upon amplification after digestion of DNA with MspI or HpaII, PCR products could not be detected in HCCs, in contrast to the normal liver and HN cases (Fig. 2), providing further evidence of the presence of unmethylated CCGG sites. Representative results of bisulfite genomic sequencing of the 5'-upstream region of the c-myc gene are shown in Figs. 1 and 3. All 33 cytosines were clearly methylated in normal liver and HNs but unmethylated in the 5 HCCs. The non-tumorous surrounding tissues of the liver at 50 and 75 weeks showed all methylated cytosines (data not shown). Results on c-myc expression from RT-PCR analysis are shown in Fig. 4. c-myc was overexpressed in HCCs but weakly expressed in normal liver and HNs. PCR products were formed proportionally to the amount of cDNA template.

The present study clearly demonstrated that HCCs, but not HNs, induced by chronic administration of the CDAA diet are characterized by a hypomethylated status of the c-myc gene, accompanied by its overexpression. It is considered that DNA methylation at specific sites can influence transcription directly by interfering with the binding of positively or negatively acting transcription factors or indirectly by the formation of inactive chromatin. DNA methylation could reduce the binding affinity of sequence-specific transcription factors. The reciprocal relation-

Fig. 3. Representative results of bisulfite genomic sequencing of samples of HCCs induced by the CDAA diet in rats. , the region of the c-myc gene inserted into the TA cloning vector; NRL, normal liver; HN, hyperplastic nodule.

Fig. 4. Representative results of the RT-PCR analysis of c-myc expression in HCCs induced by the CDAA diet in rats. M, DNA size marker; N, normal liver; HN, hyperplastic nodule. For semi-quantitative RT-PCR, cDNA solution at different concentrations (1: 0.05 μl, 2: 0.5 μl, 3: 2 μl) in 20-μl was subjected to PCR amplification, using both c-myc and GAPDH primers.
Liver carcinogenesis involves multiple steps from putative neoplastic to neoplastic lesions. It is known that the majority of nodules (over 90 to 95%) may undergo remodeling by redifferentiation to normal-appearing liver and only a small minority of nodules (1 to 3%) persist. However, biological factors that distinguish remodeling and persistent nodules have yet to be identified. It is suspected that a primary step toward the development of cancer is the acquisition of genomic instability. Furthermore, the striking correlation between genomic instability and methylation capacity implies that methylation abnormalities might play a role in chromosome segregation processes in cancer cells. Thomas has in fact proposed a hypothesis relating methylation to aneuploidy. The DNA content per nucleus in nodules is mainly euploid, unlike the aneuploidy in the later-appearing HCCs, and it was suggested that the premalignant state is not necessarily associated with major chromosomal abnormalities. The results documented here suggest that altered methylation could be a critical event for malignant transformation from HN to HCC.

In conclusion, the present study demonstrated hypomethylation of c-myc in HCCs, but not HNs, and we suggest that this might serve as a target for chemoprevention and novel therapeutic approaches. Further studies to assess changes in other genes and to clarify the linkage of methylation status and gene expression during CDAA-induced liver carcinogenesis are now required.

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line-deficient, L-amino acid-defined diet in rats. *Jpn. J. Cancer Res.*, **85**, 499–505 (1994).

7) Tsujiuchi, T., Kobayashi, E., Nakae, D., Mizumoto, Y., Andoh, N., Kitada, H., Ohashi, K., Fukuda, T., Kido, A., Tsutsumi, M., Denda, A. and Konishi, Y. Prevention by methionine of enhancement of hepatocarcinogenesis by coadministration of a choline-deficient L-amino acid-defined diet and ethionine in rats. *Jpn. J. Cancer Res.*, **86**, 1136–1142 (1995).

8) Farber, E. The step-by-step development of epithelial cancer: from phenotype to genotype. *Adv. Cancer Res.*, **70**, 21–48 (1988).

9) Tsujiuchi, T., Tsutsumi, M., Kido, A., Kobitsu, K., Takahama, M., Majima, T., Denda, A., Nakae, D. and Konishi, Y. Increased telomerase activity in hyperplastic nodules and hepatocellular carcinomas induced by a choline-deficient L-amino acid-defined diet in rats. *Jpn. J. Cancer Res.*, **87**, 1111–1115 (1996).

10) Tsujiuchi, T., Kido, A., Nakae, D., Takahama, M., Majima, T., Kobitsu, K., Okajima, E., Tsutsumi, M., Denda, A. and Konishi, Y. Infrequent Ki-ras and an absence of p53 mutations in hepatocellular carcinomas induced by a choline deficient L-amino acid defined diet in rats. *Cancer Lett.*, **108**, 137–141 (1996).

11) Cheah, M. S. C., Wallace, C. D. and Hoffman, R. M. Hypomethylation of DNA in human cancer cells: a site specific change in the c-myc oncogene. *J. Natl. Cancer Inst.*, **73**, 1057–1065 (1984).

12) Nambu, S., Inoue, K. and Sasaki, H. Site-specific hypomethylation of the c-myc oncogene in human hepatocellular carcinoma. *Jpn. J. Cancer Res. (Gann)*, **78**, 695–704 (1987).

13) Clark, S. J., Harrison, J., Paul, C. L. and Frommer, M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.*, **22**, 2990–2997 (1994).

14) Swafford, D. S., Middleton, S. K., Palmisano, W. A., Nikula, K. J., Tesfaigzi, J., Baylin, S. B., Herman, J. G. and Belinsky, S. A. Frequent aberrant methylation of p16 INK-4a in primary rat lung tumors. *Mol. Cell. Biol.*, **17**, 1366–1374 (1997).

15) Squire, R. A. and Levitt, M. H. Report of a workshop on classification of specific hepatocellular lesions in rats. *Cancer Res.*, **35**, 3214–3223 (1975).

16) Jones, P. A. and Laird, P. W. Cancer epigenetics comes of age. *Nat. Genet.*, **21**, 163–167 (1999).

17) Clark, S. J., Harrison, J. and Molloy, P. L. Sp1 binding is inhibited by 5′CpG methylation. *Gene*, **195**, 67–71 (1997).

18) Zaposik, W. F., Cronin, G. M., Lyn-Cook, B. D. and Pouirier, L. A. The onset of oncogene hypomethylation in the livers of rats fed methyl-deficient, amino acid-defined diet. *Carcinogenesis*, **13**, 1869–1872 (1992).

19) Wainfan, E. and Pouirier, L. A. Methyl groups in carcinogenesis: effects on DNA methylation and gene expression. *Cancer Res.*, **52**, 2071s–2077s (1992).

20) Dizik, M., Christman, J. K. and Wainfan, E. Alterations in expression and methylation of specific genes in livers of rats fed a cancer promoting methyl-deficient diet. *Carcinogenesis*, **7**, 1307–1312 (1991).

21) Denda, A., Rao, P. M., Rajalakshmi, S. and Sarma, D. S. R. 5-Azacytidine potentiates initiation induced by carcinogenesis in rat liver. *Carcinogenesis*, **6**, 145–146 (1985).

22) Rao, P. M., Antony, A., Rajalakshmi, S. and Sarma, D. S. R. Studies on hypomethylation of liver DNA during early stages of chemical carcinogenesis in rat liver. *Carcinogenesis*, **10**, 933–937 (1989).

23) Hartwell, L. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell*, **71**, 543–546 (1992).

24) Lengauer, C., Kinzler, K. W. and Vogelstein, B. DNA methylation and genetic instability in colorectal cancer cells. *Proc. Natl. Acad. Sci. USA*, **94**, 2545–2550 (1997).

25) Thomas, J. H. Genomic imprinting proposed as a surveillance mechanism for chromosome loss. *Proc. Natl. Acad. Sci. USA*, **92**, 480–482 (1995).

26) Becker, F. F., Fox, R. A., Klein, K. M. and Wolman, S. R. Chromosome patterns in rat hepatocytes during N-2-fluorenylacetamide carcinogenesis. *J. Natl. Cancer Inst.*, **46**, 1261–1269 (1971).