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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

Toshifumi Tsujiuchi, Masahiro Tsutsumi, Yasutaka Sasaki, Makoto Takahama and Yoichi Konishi[1]

Department of Oncological Pathology, Cancer Center, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521

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.[1)] 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.[2–4)] 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.[5, 6)] 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.[6, 7)] A critical event is the conversion of HNs, putative preneoplastic lesions, to HCCs.[8)] 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.[9, 10)] 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[11)] and HCCs.[12)]

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.[5)] Subgroups of 3 to 5 rats were killed at 50 or 75

[1] To whom requests for reprints should be addressed.
E-mail: ykonishi @ naramed-u.ac.jp

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 *Msp*I or *Hpa*II. 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.[13] Briefly, 2 μg aliquots of genomic DNA, isolated from frozen tissue by standard phenol extraction, were digested with *Eco*RI. Ten nanograms of *Eco*RI-digested genomic DNA suspended in 20 μl of water was denatured by incubation with a final concentration of 0.3 *M* NaOH at 37°C for 15 min. The resulting single-stranded DNA was sulfonated and deaminated by incubation with hydroquinone to a final concentration of 0.5 m*M* and sodium bisulfite (pH 5.0) to a final concentration of 3.1 *M*, at 55°C for 16 h, during which heat denaturation at 94°C for 5 min was performed every 3 h. Samples were purified using a desalting column (Wizard-DNA Clean-Up System, Promega, Madison, WI) and DNAs were eluted with 50 μl of water and desulfonated with a final concentration of 0.3 *M* NaOH at 37°C for 15 min. Solutions were neutralized by addition of NH_4OAc, pH 7.0, to 3 *M*, precipitated with ethanol and resuspended in water. The bisulfite-modified DNA was PCR-amplified using primers 5′-AAAAA-GAAGCGAGGGGAGGGAT-3′ (sense) and 5′-ACAAA-AATTCCCTTCCCTGCGCCT-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′-GGAACTATGACCTCGACTACGACTC-3′ (sense) and 5′-GCAGCGGCCGAGAAGCCGCTCCAC-AT-3′ (antisense); and for GAPDH, 5′-GGTGCTGAGT-ATGTCGTGGA-3′ (sense) and 5′-GCCATGCCAGT-GAGCTTCCC-3′ (antisense).[14] 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.

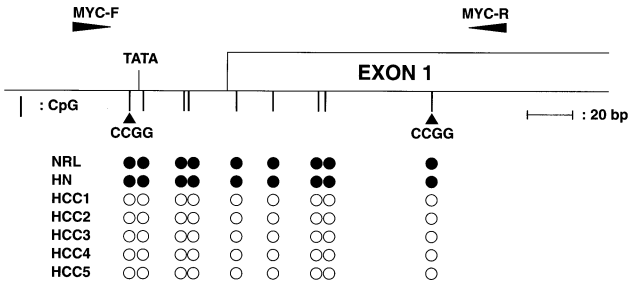

Fig. 1. The 5′ upstream region of the rat c-*myc* gene and the methylation status of CpG sites. The CpG residues that are methylated or unmethylated are indicated in each case based on the results obtained in Fig. 3. |, CpG site; CCGG, *Hpa*II site; NRL, normal liver; HN, hyperplastic nodule; ●, methylated cytosine; ○, unmethylated cytosine.

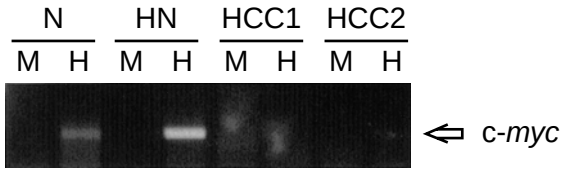

Fig. 2. Analysis of the methylation status of the 5′-upstream region of the c-*myc* gene. Amplification of the 5′-upstream region of the c-*myc* gene in a normal liver (N), a hyperplastic nodule (HN) and HCCs after digestion with the indicated restriction endonucleases. M, digested with *Msp*I; H, digested with *Hpa*II.

Histological diagnosis of HNs and HCCs was made based on the criteria described previously.[15] 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 *Msp*I or *Hpa*II, 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.[1, 16] DNA methylation could reduce the binding affinity of sequence-specific transcription factors.[16, 17] The reciprocal relation-

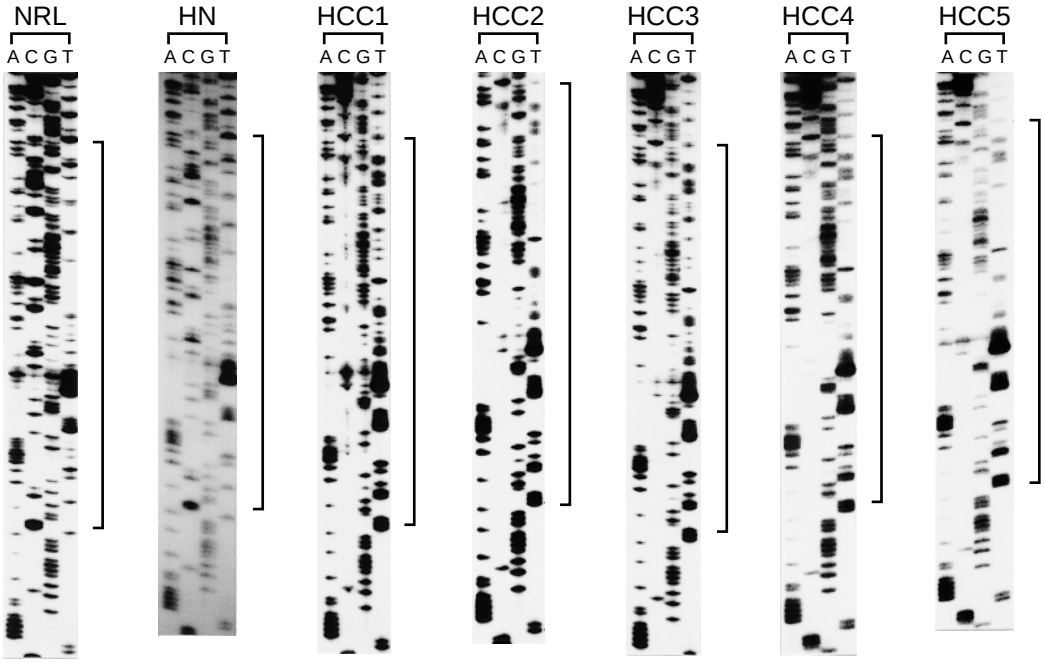

Fig. 3. Representative results of bisulfite genomic sequencing of samples of HCCs induced by the CDAA diet in rats.], the region of the c-*myc* gene inserted into the TA cloning vector; NRL, normal liver; HN, hyperplastic nodule.

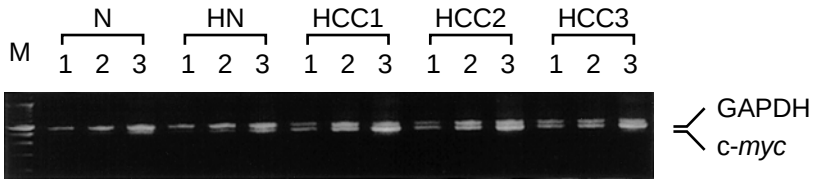

Fig. 4. Representative results of the RT-PCR analysis of c-*myc* expression in HCCs induced by the CDAA diet in rats. M, DNA size marker; N, normal liver; HN, hyperplastic nodule. For semi-quantitative RT-PCR, cDNA solution at different concentrations (1: 0.05 μl, 2: 0.5 μl, 3: 2 μl) in 20-μl was subjected to PCR amplification, using both c-*myc* and GAPDH primers.

ship between the density of methylated cytosine residues in the promoter regions and the transcription activity of a gene has been widely documented.[16]

The method routinely used for examination of aberrant methylation of oncogenes is enzyme digestion followed by Southern blot analysis.[18–20] However, this is not sufficiently sensitive to determine precise methylation patterns with only small quantities of DNA. Recently, a highly sensitive genomic sequencing technique was developed which is capable of detecting all methylated cytosines on both strands of any target sequence, using DNA isolated from fewer than 100 cells.[13] In this method, sodium bisulfite is used to convert cytosine residues to uracil residues in single-stranded DNA, under conditions where 5-methylcytosine remains non-reactive. In the present experiment, we used this sensitive method to detect hypomethylation of the 5′-upstream region, including the promoter sequence and exon 1 of the c-*myc* gene. The importance of hypomethylation of liver DNA during early stages of chemical carcinogenesis in rat liver is suggested by the fact that inhibitors of DNA methylation given after a carcinogen potentiate initiation.[21, 22] Aberration of methylation of oncogenes in the livers of rats given diethylnitrosamine (DEN) and/or methyl-deficient diet (MDD) has been studied by enzyme digestion followed by Southern blot analysis.[18, 19] MDD produced hypomethylation of c-Ha-*ras*, c-Ki-*ras* and c-*fos* at all times until 32 weeks after the beginning of the experiment, independently of DEN initiation, and the methylation changes in the c-Ha-*ras* gene increased in intensity throughout, with a similar pattern of aberrant methylation detected in preneoplastic and neoplastic nodules. Hypomethylation of the second and third exon and 3′-flanking sequences of c-*myc* gene has been detected in the liver of rats fed MDD from 1 to 4 weeks.[20] Further, hypomethylation was apparent in the 5′-flanking region and exon 1 of c-*myc* in both MDD and basal diet-fed rats. The discrepancy with the present results presumably reflects the different techniques applied, since the technique of enzyme digestion followed by Southern blot analysis is not sensitive for 188-bp sized DNA.

Liver carcinogenesis involves multiple steps from putative neoplastic to neoplastic lesions.[8] 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.[8] 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.[23] 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.[24] Thomas has in fact proposed a hypothesis relating methylation to aneuploidy.[25] 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.[26] 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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