

## Histone H3 lysine 9 and H4 lysine 20 trimethylation and the expression of Suv4-20h2 and Suv-39h1 histone methyltransferases in hepatocarcinogenesis induced by methyl deficiency in rats

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**The field of cancer epigenetics has received much attention in recent years. However, the relationship of cancer epigenetics with cancer etiology is not clear. Recent studies suggest the involvement of altered DNA methylation and histone modifications in the emergence of epigenetically reprogrammed cells with specific tumor-related phenotypes at premalignant stages of tumor development. In this study, we used a methyl-deficient model of rodent hepatocarcinogenesis to examine the roles of DNA, histone H3 lysine 9 and histone H4 lysine 20 methylation, and the level of the expression of Suv39h1 and Suv4-20h2 histone methyltransferases in the carcinogenic process. We demonstrated that the development of liver tumors was characterized by progressive demethylation of DNA repeats, decrease in histone H4 lysine 20 trimethylation, and a gradual decrease in the expression of Suv4-20h2 histone methyltransferase. A prominent increase in the trimethylation of histone H3 lysine 9 and in the expression of Suv39h1 histone methyltransferase was observed in preneoplastic nodules and liver tumors indicating the promotional role of these epigenetic alterations at later stages of carcinogenesis. The appearance of tumor-specific epigenetic alterations (demethylation of repetitive elements, loss of histone H4 lysine 20 trimethylation, altered expression of Suv4-20h2 and Suv39h1 histone methyltransferases) at preneoplastic stages of hepatocarcinogenesis provides experimental support for the epigenetic hypothesis of tumorigenesis that considers stress-induced epigenetic reprogramming of the cell as an important prerequisite to succeeding mutations.**

### Introduction

Cancer cells are characterized by prominent epigenetic dysregulation, including altered DNA methylation pattern, chromatin modification, and loss of genomic imprinting.

**Abbreviations:** methyl-deficient diet (MDD); Histone methyltransferases (HMTs); long interspersed nucleotide elements (LINE-1); chromatin immunoprecipitation (ChIP); short interspersed nuclear elements (SINE).

Despite the fact that the field of cancer epigenetics has received much attention in recent years, the area regarding the relationship of cancer epigenetics to cancer etiology is mainly overlooked (1). The induction of hepatocarcinogenesis by methyl-deficient diet (MDD) in rodents is well documented in literature (2–4). It is believed that aberrant epigenetic alterations imposed by this diet may be one of the main mechanisms responsible for malignant transformation of rat liver cells (5–8). Progressive demethylation of cytosine residues at CpG dinucleotides in the genome as a whole and in the promoters of specific proto-oncogenes has been reported in several studies of the methyl-deficient rat model of hepatocarcinogenesis (5–8). Despite the fact that global and regional DNA hypomethylation are the main epigenetic alterations observed in this model, recent studies have also shown that hypermethylation of some tumor-suppressor genes (e.g. *p16<sup>INK4A</sup>*, *p53*, and *PTPRO*; 9–11), and the induction of enzymes involved in DNA methylation (7,12) contribute to the formation of altered epigenetic phenotype. Similar epigenetic dysregulation is observed in many other cancers (13–19) and in response to stressful environments (20). Specifically, progressive loss of cytosine methylation in the genome was demonstrated in many other human cancer cells (13–15,19,21). This phenotype was also induced experimentally by different carcinogens and often took place at premalignant stages of tumor development (20,22–24).

There is a tight interaction between DNA methylation and modifications of histones (25–27). Recent studies have revealed genetic links between methylation of DNA and trimethylation at lysine 9 of histone H3 (H3-Lys9) and at lysine 20 of histone H4 (H4-Lys20) in organisms as diverse as fungi, plants, mice and humans (25–28). These modifications of histones selectively occur in pericentromeric heterochromatin (28–30) and are considered to be indicators of repressive chromatin states. Histone methyltransferases (HMTs) Suv39h1 and Suv4-20h2 are believed to be responsible for silencing of heterochromatin by acting as the enzymes responsible for trimethylation of histone H3-Lys9 and histone H4-Lys20 (19,29,31,32). According to a recent study by Fraga *et al.* (19), the loss of histone H4-Lys20 trimethylation occurs early during the tumorigenic process in human cells and is associated with the hypomethylation of DNA repetitive sequences.

Considering these findings, we hypothesized that loss of CpG methylation imposed by MDD may lead to demethylation of pericentromeric heterochromatin and, therefore, to demethylation of repetitive sequences, alteration in histone H3-Lys9, histone H4-Lys20 trimethylation, and in the activity of HMTs Suv39h1 and Suv4-20h2. To investigate this hypothesis, we have examined the methylation status of repetitive DNA elements, the status of histone H3-Lys9 and H4-Lys20 methylation, and the level of the expression of Suv39h1 and Suv4-20h2 HMTs in the methyl-deficient rat model of hepatocarcinogenesis.

## Materials and methods

### *Animals, diets and tissue preparations*

Male weanling F344 rats were obtained from the National Center for Toxicological Research breeding facility, housed 2 per cage in a temperature-controlled (24°C) room with a 12 hour light–dark cycle, and given *ad libitum* access to water and NIH-31 pelleted diet (Purina Mills, Richmond, IN). At four weeks of age, the rats (body weight 50g) were allocated randomly to receive either a low methionine (0.18%) diet, lacking in choline and folic acid (Dyets, Inc, Bethlehem, PA), or a control diet supplemented with 0.4% methionine, 0.3% choline, and 2 mg/kg folic acid. Diets were stored at 4°C and given *ad libitum* with biweekly replacement. Body weights and food consumption were recorded weekly. No significant differences in body weights between diet groups were observed. Six rats per diet group and four rats per control group were killed at 36 and 54 weeks after diet initiation. These time points represent, respectively, the irreversible preneoplastic and tumor stages in the development of liver cancer (9). The livers were excised, frozen immediately in liquid nitrogen, and stored at –80°C for subsequent analyses. Individual nodules and tumors were dissected from livers prior to freezing in liquid nitrogen.

### *Determination of methylation status of identifier (ID) elements by methylation-sensitive McrBC–PCR assay*

Genomic DNA (1 µg) was digested with 20 units of McrBC endonuclease (New England Biolabs, Beverly, MA) for 2 hrs at 37°C. Undigested DNA served as control. McrBC is an endonuclease, which, contrary to methylation-sensitive restriction endonucleases, cleaves DNA containing 5-methylcytosine on one or both strands, but will not act on unmethylated DNA (33,34). Cleavage of methylated DNA by McrBC induces DNA strand breaks and abrogates PCR amplification (35). Conversely, the presence of unmethylated cytosines in DNA prevents enzyme cleavage and can be detected by PCR amplification product recovery. Following McrBC treatment, subsequent PCR was used to amplify rat ID elements. Each PCR reaction contained 0.25 µg of undigested or McrBC-digested DNA and 50 pmol of each primer in 25 µl of 1X AmpliTaq Gold Master Mix (Applied Biosystems, Foster City, CA). The sequence of primers has been previously described (36). The cycling conditions consisted of an initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 60 s, and extension at 72°C for 60 s. The semi-quantitative aspect of the procedure was verified by a linear increase in PCR product recovery with increasing cycle number and DNA template concentration. The PCR products were separated on 2% agarose gels, stained with ethidium bromide, photographed, and the band intensity was analyzed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The results are presented as ratio of PCR product recovery after digestion of DNA with McrBC to undigested DNA.

### *Determination of methylation status of long interspersed nucleotide elements (LINE-1) by COBRA-assay*

Methylation status of LINE-1 was determined by COBRA assay, which consists of a standard bisulfite modification of genomic DNA, subsequent PCR amplification and digestion of PCR product with appropriate restriction endonuclease (37–39). The combination of sodium bisulfite treatment and PCR amplification results in methylation-dependent creation of new restriction endonuclease sites, such as RsaI, or methylation-dependent retention of pre-existing sites, such as BstUI. Briefly, 2 µg of genomic DNA was treated with sodium bisulfite as previously described in detail (40). Bisulfite-modified DNA was PCR-amplified with primers corresponding to the regulatory region of rat LINE-1 sequence (39). The sense primer was 5'-TTTGGTGAGTTTGGGATA-3', and the antisense primer was 5'-CTCAAAAATACCCACCTAAC-3'. The PCR amplification consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 60 s, and extension at 72°C for 60 s. The PCR products were digested with 20 units of RsaI or BstUI restriction endonuclease (New England Biolabs) for 16 hrs, respectively. The digested PCR products were separated on 3% high resolution agarose gels (Sigma, St Louis, MO), stained with ethidium bromide, photographed, and the band intensity was analyzed by ImageQuant software (Molecular Dynamics).

### *Histone extraction*

The acid cell extracts were prepared from frozen liver tissues using lysis buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 1.5 mM PMSF, followed by the addition of HCl to a final concentration of 200 mM according to manufacturer's protocol (Upstate, Charlottesville, VA). Cell lysates were centrifuged at 14 000 ×g for 10 min at 4°C, and the acid-insoluble pellets were discarded. The supernatant fractions, which contain the acid-soluble proteins, were purified by sequential dialysis against 100 mM acetic acid and H<sub>2</sub>O. Protein concentrations were determined by Bradford assay (Pierce, Rockford, IL). Aliquots of total histones were stored at –80°C.

### *Western blot analysis of histone methylation*

Equal amount of total histones (40 µg) were mixed with two volumes of gel loading buffer (250 mM Tris–HCl, pH 8.0, 20% β-mercaptoethanol, 40% glycerol, 8% SDS, 1.2 mg/ml bromophenol blue), heated for 5 min at 95°C, and resolved on 15% polyacrylamide gel. Proteins were transferred onto PVDF membranes. The membranes were blocked for 4 h in TBS buffer containing 5% nonfat dry milk and 0.1% Tween-20. Anti-trimethyl-histone H3-Lys9 and anti-trimethyl-histone H4-Lys20 primary antibodies were diluted 1:1000 and 1:2000, respectively, according to manufacturer's recommendations (Upstate). Primary antibody binding was performed at 4°C overnight with constant shaking. Secondary donkey anti-rabbit antibodies labeled with alkaline phosphatase (Santa Cruz Biotechnology, Santa Cruz, CA) were applied at 1:5000 dilutions, and binding was carried out at room temperature for 1.5 h. Chemifluorescence detection was performed with the ECF Substrate for Western Blotting (Amersham Biosciences, Piscataway, NJ) and detected directly by Storm Imaging System (Molecular Dynamics). Images were representative of three independent immunoblots and were analyzed by ImageQuant software. All membranes were stained with Coomassie Blue and with anti-histone H3 and anti-histone H4 primary antibodies to confirm equal protein loading.

### *Chromatin immunoprecipitation (ChIP) assay for LINE-1-associated histone methylation*

Formaldehyde cross-linking and ChIP assays were performed using Chromatin Immunoprecipitation Assay Kit (Upstate) as previously described in detail (41). Chromatin was immunoprecipitated with anti-trimethyl-histone H3-Lys9 or anti-trimethyl-histone H4-Lys20 primary antibodies (Upstate) or carried out through the protocol with no antibody added as a control (mock precipitation). Purified DNA from immunoprecipitates was analyzed by PCR using primer pair, sense 5'-TTGGGACACCGGAAGCAGAATTTC-3' and antisense 5'-GTTTCCTCTTGGGCCAGGGATGA-3' designed to amplify the 220 bp fragment of the regulatory region of rat LINE-1 sequence (40). All PCR amplifications were performed in a total volume of 50 µl with 5 µl of immunoprecipitated DNA. An input DNA sample or a no-antibody control was also analyzed by PCR. The PCR was optimized with input DNA and semi-quantitative aspect of the procedure was verified by a linear increase in PCR product recovery with increasing cycle number and input DNA template concentration. The PCR products were separated on 2% agarose gels, stained with ethidium bromide, photographed, and the band intensity was analyzed by ImageQuant software (Molecular Dynamics).

### *Western blot analysis of Suv39h1 and Suv2-40h2 HMTs expression*

Liver tissue lysates were prepared by homogenization of 50 mg of tissue in 500 µl of lysis buffer (50 mM Tris–HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 µg/ml each aprotinin, leupeptin, pepstatin; 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF), sonication and incubation at 4°C for 30 min followed by centrifugation at 10 000 ×g at 4°C for 20 min.

Extracts containing equal quantities of proteins were separated on 10% polyacrylamide gel and transferred to PVDF membranes. Membranes were probed with antibodies specific for Suv39h1 (Upstate) and Suv2-40h2 (Abcam, Cambridge, MA) HMTs at 1:1000 and 1:1500 dilutions, respectively. Alkaline phosphatase-coupled donkey anti-rabbit secondary antibodies were used for visualization.

### *Statistical analysis*

Results are presented as mean ±SD. Statistical analyses were conducted by two-way ANOVA using the Sigmastat software (Jandel Scientific, San Rafael, CA).

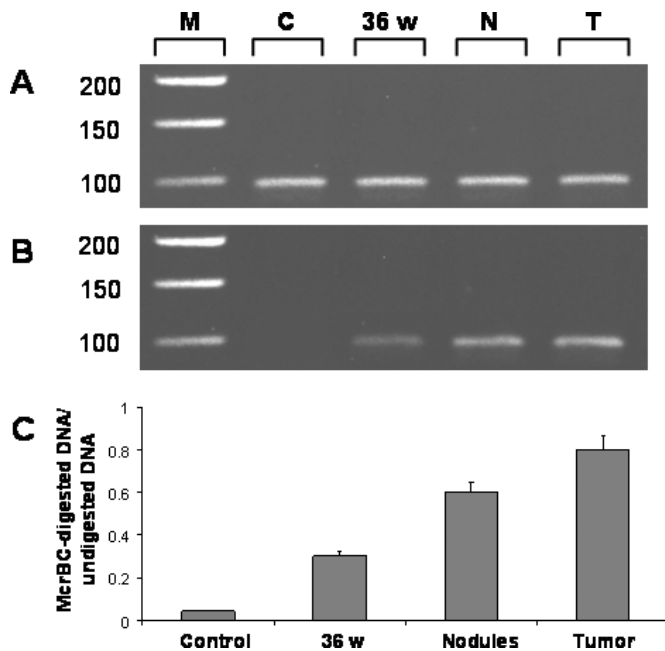
## Results

### *Hypomethylation of ID elements and LINE-1 in preneoplastic livers and liver tumors*

In the previous studies examining the methyl-deficient model of hepatocarcinogenesis we showed the importance of global DNA hypomethylation in the development of liver tumors (7,8). It is known that majority of cytosine methylation in mammals is located in repetitive DNA elements (42), and that most of this methylation resides in transposons (42,43), which are interspersed repeated sequences that constitute almost 50% of human genome (44), and about 40% of rat genome (45). It has been suggested that genome-wide DNA

hypomethylation in cancer cells largely affects transposons (42). In view of this, we measured the methylation status of ID elements, which are a major family of short interspersed nuclear elements (SINE) widely distributed in the rat genome (36,45), and LINE-1, which constitute almost 23% of rat genome (45) at various stages of rat hepatocarcinogenesis. In order to determine the methylation status of ID elements in the liver during hepatocarcinogenesis, we used a methylation-sensitive McrBC-PCR assay, which is based on the ability of McrBC to degrade heavily methylated DNA but not to affect unmethylated DNA or DNA methylated at one or several sites (33,34). Subsequent PCR allows determination of the methylation status of ID elements because cleavage of methylated DNA by McrBC induces DNA strand breaks and abrogates PCR amplification. Conversely, presence of unmethylated cytosines in DNA prevents enzyme cleavage and can be detected by PCR amplification product recovery. Figure 1 shows the representative photograph of agarose gel of amplified rat ID elements using undigested (Panel A) or McrBC-digested DNA (Panel B) templates. Complete absence of PCR product after pretreatment of DNA with McrBC indicates the substantial methylation level of ID elements in liver of control rats. In contrast, a gradual increase in PCR product recovery after McrBC digestion of DNA confirms the hypomethylated status of ID elements in preneoplastic livers and liver tumors induced by methyl deficiency.

Figure 2 shows the status of LINE-1 methylation in control livers, preneoplastic livers and liver tumors, as determined by

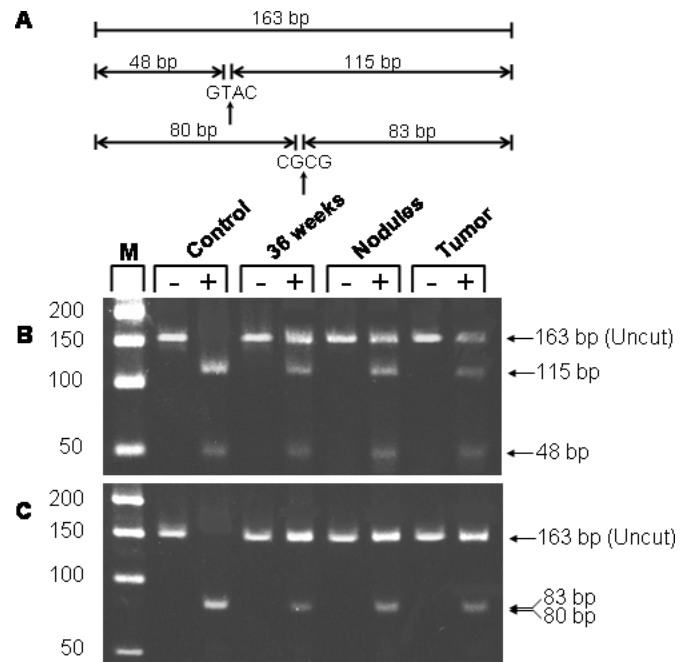


**Fig. 1.** Methylation status of ID elements in control livers, preneoplastic livers and liver tumors as detected by methylation-sensitive McrBC-PCR assay. (A) Representative photograph of PCR amplification of the rat ID elements when undigested genomic DNA was used as a template. PCR amplification of the rat ID core domain yielded three bands, 98, 330 and 690 bp in size. However, only 98 bp fragment shows 96–100% homology to the ID core domain (34). (B) Representative photograph of PCR amplification when McrBC-digested genomic DNA was used as a template. (C) Ratio between McrBC-digested DNA and undigested DNA. For each PCR we used 250 ng of undigested or McrBC-digested DNA as templates. Increase in McrBC-digested DNA/undigested DNA ratio is indicative of hypomethylation status of ID elements. The results are mean data of four animals  $\pm$  SD.

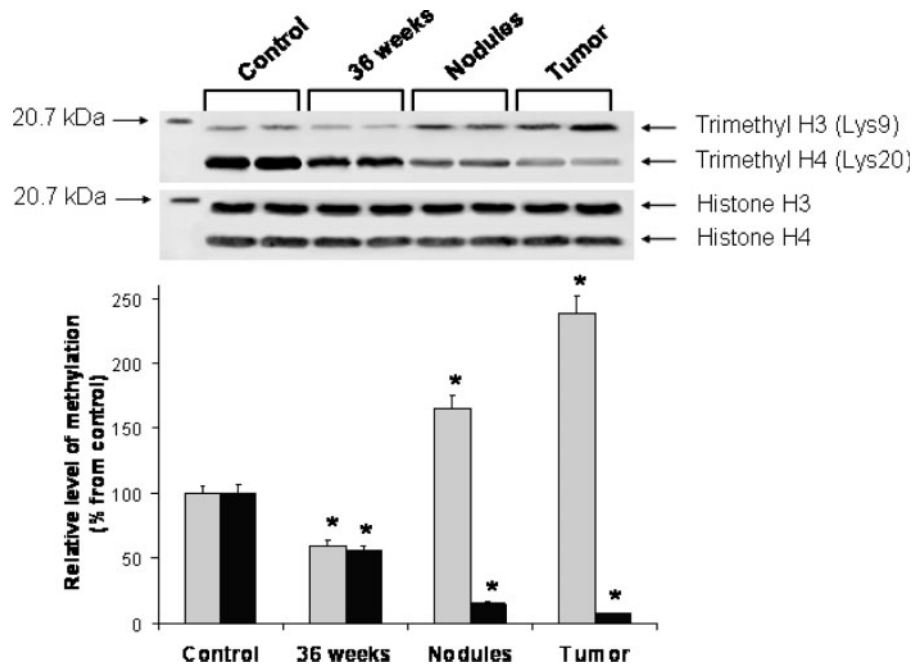
COBRA assay. The complete digestion of PCR products by RsaI (Panel B) and BstUI (Panel C) restriction endonuclease confirms the existence of methylation in regulatory region of LINE-1 in liver of control rats. In contrast, the presence of undigested PCR products after RsaI and BstUI treatment in preneoplastic livers and tumors shows the loss of LINE-1 methylation because cleavage would occur only if the CpG dinucleotides within RsaI and BstUI recognition sequences are methylated.

#### *Progressive loss of histone H4-Lys 20 trimethylation accompanied by increased histone H3-Lys9 trimethylation*

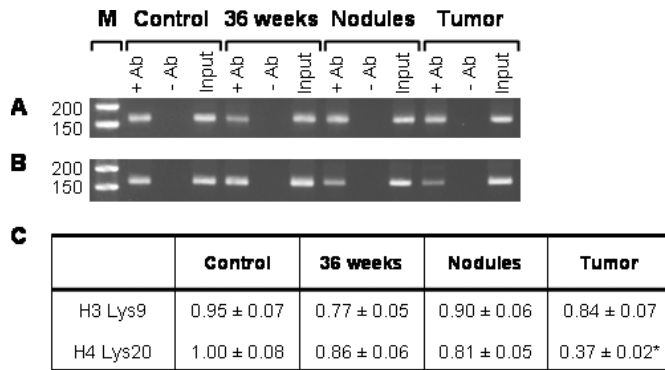
Taking into consideration the results of recent studies that have shown aberrant histone modifications play an important role in carcinogenesis (25), we examined the alterations of histone H4-Lys20 and histone H3-Lys9 trimethylation during hepatocarcinogenesis. Figure 3 shows that development of liver tumors is characterized by progressive changes in methylation of histone H3-Lys9 and H4-Lys20. After 36 weeks of methyl deficiency, level of trimethylation of histone H3-Lys9 and histone H4-Lys20 were decreased by 40% and 44%, respectively, compared with the age-matched control rats. At this time point, liver tissue of methyl-deficient rats contains substantial number of placental isoform of glutathione-S-transferase (GST $\pi$ )-positive foci as detected by immunohistochemical staining (46). The accumulated evidence suggests that the presence of enzyme-altered foci in liver is a sensitive marker for initiated cells and represents precursor lesions, which are



**Fig. 2.** Methylation status of LINE-1 in control livers, preneoplastic livers and liver tumors as detected by COBRA assay. (A) Diagram of the PCR product of rat LINE-1 regulatory region (39). The location of RsaI and BstUI sites analyzed for methylation status by COBRA is shown. (B) Representative photograph shows a COBRA assay with the RsaI restriction endonuclease. (C) Representative photograph shows a COBRA assay with the BstUI restriction endonuclease. Complete digestion of PCR product in control animals indicates the substantial methylation level of LINE-1 at RsaI and BstUI sites. Cleavage will occur only if the CpG sequences have been retained during the bisulfite conversion. Presence of uncut (163 bp) PCR products illustrates the hypomethylation status of LINE-1 in preneoplastic livers and tumors.



**Fig. 3.** Western blot analysis of methylation status of histone H3-Lys9 and histone H4-Lys20. Acid extracts of total histones were separated by SDS-PAGE and subjected to immunoblotting using specific antibodies against trimethyl histone H3-Lys9 and trimethyl histone H4-Lys20. Results are presented as change in methylation relative to control animals and shown as the mean  $\pm$  SD. Gray bars—trimethyl histone H3-Lys9; black bars—trimethyl histone H4-Lys20. Equal sample loading was confirmed by immunostaining against histone H3 and histone H4. These results were reproduced in two independent experiments. Representative immunoblot images are shown. \*Significantly different from control.



**Fig. 4.** ChIP analysis of LINE-1-associated histone H3-Lys9 and H4-Lys20 trimethylation states in control livers, preneoplastic livers and liver tumors. ChIP was performed with anti-trimethyl-histone H3-Lys9 (A) and anti-trimethyl-histone H4-Lys20 (B) primary antibodies. Each sample was incubated in the presence (+) or absence (-) of antibodies. Purified DNA from each immunoprecipitated sample was analyzed by PCR with primers specific for regulatory region of LINE-1. A sample from total input chromatin (Input) was used as positive control for each PCR. Ethidium bromide-stained PCR products are shown. These results were reproduced in two independent experiments, with a representative data set shown. (C) Quantitative analyses of the H3-Lys9 and H4-Lys20 trimethylation at the Line-1 regulatory region. The data are presented as bound-input ratio. \*Significantly different from control.

causally related to carcinogenic process in the liver (47,48). The prominent decrease of trimethylation of histone H4-Lys20 was detected in nodules and tumors, and corresponded to 40 and 20% of control level, respectively. Consistent with this global histone H4-Lys20 trimethylation depletion, ChIP analysis of LINE-1 also shows a loss of H4-Lys20 trimethylation at LINE-1 regulatory region that becomes statistically significant in tumors (Figure 4). In contrast, preneoplastic nodules

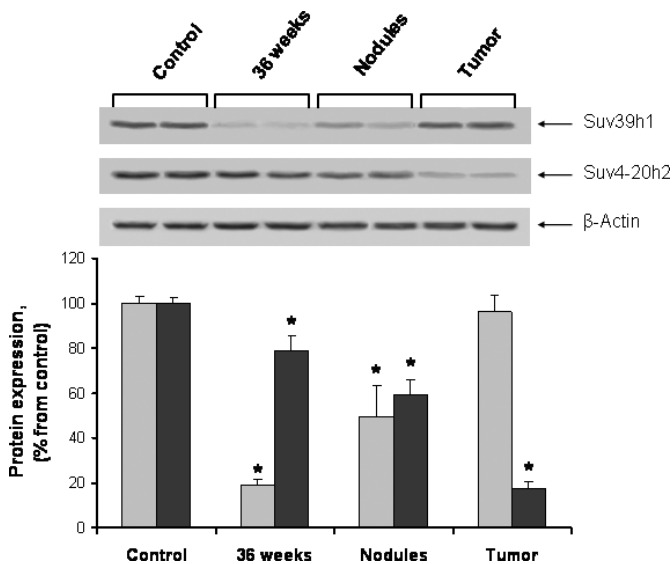
and liver tumors displayed the prominent increase in global trimethylation of histone H3-Lys9.

#### *Different expression pattern of Suv4-20h2 and Suv39h1 HMTs during hepatocarcinogenesis*

In order to determine the possible mechanism of alterations of histone H4-Lys20 and H3-Lys9 trimethylation, we analyzed the expression of Suv4-20h2 and Suv39h1 HMTs in liver tissue during hepatocarcinogenesis. Results of recent experiments demonstrate that Suv4-20h2 HMT is the main contributor to trimethylation of histone H4 Lys20 (19,28,32) and Suv39h1 HMT is responsible for trimethylation of histone H3-Lys9 (28,29). In addition to methylation of histone H3-Lys9, Suv39h1 HMT may also be involved in the recruitment of Suv4-20h2 HMT to pericentromeric regions (28). Expression of Suv4-20h2 HMT steadily decreased along with development of liver tumors, and reached its lowest level in tumor tissue (Figure 5). The opposite trend was observed in the expression of Suv39h1: the lowest level of expression was detected in liver tissue after 36 weeks of deficiency, and after that there was a gradual increase in expression in preneoplastic nodules and tumors.

#### Discussion

The results of the study showed that progressive loss of CpG methylation imposed by MDD occurred at repetitive ID elements and LINE-1 and was accompanied by a decrease in histone H4-Lys20 trimethylation. This was evidenced by a progressive loss of trimethylation of histone H4-Lys20 at preneoplastic and tumor stages of liver cancer development and a corresponding decrease in the expression of Suv4-20h2 HMT. In both cases, there was a linear decline in the values from control to premalignant stage (36 weeks), nodules and



**Fig. 5.** Expression of Suv39h1 and Suv4-20h2 histone methyltransferases. Liver tissue lysates were separated by SDS-PAGE and subjected to immunoblotting using specific antibodies against Suv39h1 and Suv4-20h2. Protein levels are presented as relative to control animals after normalization to  $\beta$ -actin and shown as the mean  $\pm$  SD. Gray bars- Suv39h1; black bars-Suv4-20h2. These results were reproduced in two independent experiments. Representative immunoblot images are shown. \*Significantly different from control.

tumor stage (56 weeks), with a consistent drop of methylation/expression by about 20–40% at each subsequent stage, and with statistically significant correlation between the parameters to be equal 0.90 ( $P < 0.05$ ). A similar observation of progressive hypomethylation of LINE-1 in liver was found in hepatocarcinogenesis induced by chronic feeding of a choline-deficient diet to rats (39).

The pattern of changes in demethylation of the repeats was consistent with linear decline in trimethylation of histone H4-Lys20 and with the diminished expression of Suv4-20h2 HMT, which is the main contributor to trimethylation of histone H4-Lys20 (19,28,31,32). There was significant correlation of the level of methylation of ID elements with both parameters ( $r = -0.98$  and  $-0.97$ , respectively;  $P < 0.05$ ). It allows us to suggest that the progressive demethylation and activation of interspersed repeats imposed by MDD is consistent with demethylation of heterochromatin that may depend on the activity of Suv4-20h2 HMT.

Emerging evidence suggests an important role of Suv39h1, Suv4-20h2 HMTs and histone H3-Lys9 and histone H4-Lys20 trimethylation in tumor suppression and in the maintenance of the genomic stability (28,32,49–51). Genetic inactivation of Suv39h1 and/or Suv4-20h2 HMTs resulted in genomic and chromosomal instabilities in a variety of somatic cells, and was associated with increased tumor risk (50,51). In mice, inactivation of Suv39h1 HMT led to development of aggressive lymphomas in response to oncogenic Ras (51). Loss of trimethylation of histone H3-Lys9 and H4-Lys20, demethylation and activation of DNA repeats in preneoplastic liver tissue after 36 weeks of methyl deficiency may subsequently lead to activation of proto-oncogenes (6) and a variety of mutational effects including chromosomal instability, DNA uncoiling, heterochromatin decondensation, DNA breakage, repeats retrotransposition, generation pseudogenes and chimeric retrotranscripts, insertions and deleterious mutations

(43,52). A recent observation concerning the causative role of DNA hypomethylation in development of aggressive T-cell lymphomas and liver tumors in mice (53,54) may also be directly related to demethylation of interspersed repeated sequences (43).

According to the epigenetic hypothesis of carcinogenesis, the emergence of epigenetically reprogrammed cells with specific tumor-related epigenetic alterations (inhibition of cell-cycle arrest and apoptosis, activation of cell proliferation) likely impacts the origin of cancer leading to succeeding mutations and malignant transformations (1,20,55). Indeed, the results of our study show that the appearance of tumor-specific epigenetic alterations (demethylation of ID and LINE-1 elements, loss of histone H4-Lys20 and H3-Lys9 trimethylation, decreased expression of Suv4-20h2 and Suv39h1 HMTs) in liver at preneoplastic stages of hepatocarcinogenesis supports the epigenetic hypothesis of carcinogenesis. Importantly, these epigenetic changes were shown previously to be associated with the formation of phenotypically GST $\pi$ -altered cell population in liver tissue (46–48).

A recent finding by Schotta *et al.* (28) established trimethylation of histone H4-Lys20 as a third component of repressive histone lysine methyl marks in pericentromeric heterochromatin in addition to trimethylation H3-Lys9 and H3-Lys27. Substantial loss of histone H4-Lys20 trimethylation and accompanied decreased expression of Suv4-20h2 HMT in preneoplastic nodules and tumors could result in a more ‘relaxed’ heterochromatin organization, which markedly impairs genome stability and severely impairs viability of cells (28,32). In light of these considerations, the increased global and LINE-1-associated trimethylation of histone H3-Lys9 accompanied by elevated level of Suv39h1 HMT in preneoplastic nodules and tumors (Figures 3, 4A and 5) may be a cellular defense mechanism for maintenance heterochromatin organization and cell viability. Dual cellular functions of Suv39h1 relating to proliferation and differentiation allow us to explain the elevated expression of this HMT in nodules and liver tumors found in the study. Besides tumor-suppressive silencing at early stages of carcinogenesis, Suv39h1 can assist in tumor progression by controlling the choice between growth and differentiation at later stages of tumor development. Considering the suppressive and promotional role of Suv39h1 on tumor development, we can suggest the following explanation for the pattern of its expression in the study (Figure 5). The initial decrease in the expression of the enzyme may be result of reduced S-adenosylmethionine/S-adenosylhomocysteine (SAM/SAH) ratio induced by MDD (51). The subsequent increase in the expression may be caused by increased requirement of the enzyme to favor proliferation over differentiation.

In conclusion, feeding MDD resulted in progressive demethylation of DNA repetitive sequences, a decrease in H4-Lys20 trimethylation and diminished expression of Suv4-20h2 histone methyltransferase. In spite of the chronic nature of the methyl deficiency, a decrease in the expression of Suv39h1 and loss of the trimethylation of histone H3-Lys9 was observed only at early premalignant stage of tumor development. In nodules and tumors there was a dramatic increase of these parameters indicating intensive epigenetic reprogramming of the genome by modification of histones after 36 weeks of MDD. These results are consistent with the epigenetic hypothesis of tumorigenesis (1,20,55), considering the carcinogenic process as an adaptation of mammalian cells

to environmental stress by epigenetic alterations and succeeding subsequent mutations, and recent evidence of the causative role of DNA hypomethylation in the development of multifocal hepatocellular carcinomas (50).

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