Genetic and epigenetic changes in fibrosis-associated hepatocarcinogenesis in mice

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Abstract

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers and is rising in incidence worldwide. The molecular mechanisms leading to the development of HCC are complex and include both genetic and epigenetic events. To determine the relative contribution of these alterations in liver tumorigenesis, we evaluated epigenetic modifications at both global and gene specific levels, as well as the mutational profile of genes commonly altered in liver tumors. A mouse model of fibrosis-associated liver cancer that was designed to emulate cirrhotic liver, a prevailing disease state observed in most humans with HCC, was used. Tumor and non-tumor liver samples from B6C3F1 mice treated with *N*-nitrosodiethylamine (DEN; a single *ip* injection of 1 mg/kg at 14 days of age) and carbon tetrachloride (CCl$_4$; 0.2 ml/kg, 2 times/week *ip* starting at 8 weeks of age for 14 weeks), as well as corresponding vehicle control animals, were analyzed for genetic and epigenetic alterations. *H-ras*, *Ctnnb1*, and *Hnf1*α genes were not mutated in tumors in mice treated with DEN+CCl$_4$. In contrast, the increased tumor incidence in mice treated with DEN+CCl$_4$ was associated with marked epigenetic changes in liver tumors and non-tumor liver tissue, including demethylation of genomic DNA and repetitive elements, a decrease in histone 3 lysine 9 trimethylation (H3K9me3), and promoter hypermethylation and functional down-regulation of *Riz1,* a histone lysine methyltransferase tumor suppressor gene. Additionally, the reduction in H3K9me3 was accompanied by increased expression of long interspersed nucleotide elements (LINE) 1 and short interspersed nucleotide elements (SINE) B2, which is an indication of genomic instability. In summary, our results suggest that epigenetic events, rather than mutations in known cancer-related genes, play a prominent role in increased incidence of liver tumors in this mouse model of fibrosis-associated liver cancer.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent, life-threatening human cancers[1]. While the overall cancer incidence and death rates are steadily declining, the incidence of HCC continues to increase[1,2]. The development and progression of HCC is a multistep process characterized by the progressive, sequential evolution of morphologically distinct pre-neoplastic lesions (formed as a result of chronic liver injury, inflammation, hepatocellular degeneration and necrosis, hepatocellular regeneration and small cell dysplasia, followed by the appearance of low- and high-grade dysplastic nodules), which eventually culminates in the formation of HCC[3,4]. In humans, 70–90% of HCC cases are associated with advanced liver fibrosis or cirrhosis[3]. HCC most often arises in the presence of chronic liver inflammation and fibrosis/cirrhosis that may result from disturbances in metabolism, toxic insults, or viral infection[5].

While the histopathologic features of HCC is well established, the molecular mechanisms of the cancer-promoting effects of the main etiological factors, including cirrhosis, are not well understood[4,6]. Elucidating the molecular mechanisms underlying the pathogenesis and progression of HCC is critical for prevention of this disease and development of effective therapies[7]. Investigation of these mechanisms using human HCC samples is desirable; however, few epidemiological studies have established both the causality and molecular underpinnings of the disease. Animal models that resemble human HCC development may provide important additional clues regarding the molecular sequelae of etiological factors linked to HCC[8]. A commonly used mouse liver cancer model is a single low dose injection of the genotoxic carcinogen N-nitrosodiethylamine (DEN) into 14-day-old male mice[9]. In addition, repeat dosing of the pro-fibrogenic agent carbon tetrachloride (CCl_4) also results in development of HCC[10]. To model key pathophysiological events of human cirrhosis-associated hepatocarcinogenesis, we used a combination of genotoxic, e.g., DEN, and non-genotoxic, e.g., CCl_4, insults to study the mechanisms of the development of fibrosis-promoted HCC In mice[11]. The incidence of liver adenomas and carcinomas was more than two-fold greater in mice treated with DEN+CCl_4 as compared to that in mice treated with each agent alone[11].

The development of HCC is driven by the accumulation of genetic and epigenetic alterations[12–14], however, a comparative analysis of the relative contribution of these aberrations is typically not performed in individual studies. The goal of the present study was to investigate the mechanistic roles and contribution of genetic and epigenetic events in fibrosis-associated liver carcinogenesis in mice. We hypothesized that distinct differences in such alterations exist in the liver tissue of animals with fibrosis-associated cancer in comparison to liver tissue of vehicle-control animals. Such alterations may explain, in part, the increased incidence of hepatic tumors in fibrotic liver in this mouse model, and provide insight into the molecular characteristics of human HCC.

Materials and Methods

Animals, treatment, and tissue preparation

The in-life portion of this study, mouse treatments, tissue collection protocols, and the incidence of neoplastic liver lesions are detailed in Uehara *et al.*[11]. Briefly, male B6C3F1/J mice were allocated randomly to one control and three experimental groups. At two weeks of age, mice from two of the experimental groups were injected *i.p.* with DEN (1 mg/kg) in sterile phosphate buffered saline (PBS; 15 ml/kg). Mice from the control group and the remaining experimental group were injected with sterile PBS only. At eight weeks of age, mice from the control and the DEN-treated groups were injected *i.p.* two times per week with sterile olive oil (15 ml/kg). Mice from the remaining two experimental groups were injected *i.p.* two times per week with CCl_4 (0.2 ml/kg) diluted in sterile olive oil for an additional 14 weeks. In summary, the groups were treated with either PBS+olive oil, DEN +olive oil, PBS+CCl_4, or DEN+CCl_4. All mice were sacrificed at 22 weeks of age.

Screening for mutations in H-ras and Ctnnb1 genes

Total DNA was isolated from frozen liver tissue samples using DNeasy Mini Kits (Qiagen, Valencia, CA) according to the manufacturer's instructions. Nested PCR analysis was used to examine codon 61 in exon 2 of the v-Ha-ras Harvey rat sarcoma viral oncogene homolog (*H-ras*) gene and codons 5–57 in exon 2 of the catenin (cadherin associated protein) beta 1 (*Ctnnb1*) gene. Reactions were performed on tumor and matched adjacent non-tumor liver tissue samples from mice treated with DEN+CCl_4, as well as normal livers from control mice. PCR products were purified, cycled with Terminal Ready Reaction Mix-Big Dye (Perkin Elmer, Foster City, CA), and sequenced.

Immunohistochemical staining for β-catenin activation and Hnf1α inactivation

The activity and cellular location of β-catenin (CTNNB1) was evaluated by immunostaining using goat polyclonal CTNNB1 (sc-1496, 1:50; Santa Cruz Biotechnology, Santa Cruz, CA) as described in Hoenerhoff *et al*[14]. The levels of glutamine synthetase (GLUL) and liver fatty acid binding protein 1 (FABP1) was assessed by immunostaining as described in Jeannot *et al.*[15].

Global DNA methylation

The methylation status of genomic DNA was evaluated by a methylation sensitive cytosine extension as described in Pogribny *et al.*[16].

McrBC-methylation sensitive quantitative PCR

The methylation status of long interspersed nucleotide elements (LINE) 1 and short interspersed nucleotide elements (SINE) B2 repetitive sequences was determined by a McrBC-methylation sensitive quantitative PCR assay as described in Martens *et al.*[17].

Methylation-specific PCR

The methylation status of CpG sites located within the promoter/first exon region of cyclin-dependent kinase inhibitor 2A (*Cdkn2a),* O^6-methylguanine-DNA methyltransferase

(*Mgmt*), suppressor of cytokine signaling 1 *(Socs1),* cadherin 1 *(Cdh1)*, and PR domain containing 2, with ZNF domain (*Riz1*) was determined by methylation-specific PCR (MSP) as previously described[18].

RNA extraction and quantitative real-time reverse transcription-PCR

Total RNA was extracted from liver tissue using TRI Reagent (Ambion, Austin, TX) according to the manufacturer's instructions. Total RNA (5 μg) was reverse transcribed and gene expression was then determined by quantitative reverse-transcription PCR (qRT-PCR) using gene expression assays (Applied Biosystems, Foster City, CA). Each sample was analyzed in duplicate. Reactions were performed in a 96-well plate format. Each plate contained one experimental gene and the housekeeping gene (*Gusb1* or *Gapdh*). The relative amount of each mRNA transcript was determined using the $2^{-\Delta\Delta Ct}$ method[19].

Western blotting

The level of trimethylation of histone 3 lysine 9 (H3K9), histone 3 lysine 27 (H3K27), and histone 4 lysine 20 (H4K20) in the livers of the mice was analyzed by Western blot analysis as described in Tryndyak *et al.*[20].

Chromatin immunoprecipitation (ChIP) assay

Formaldehyde cross-linking and ChIP assay with primary antibodies against H3K9me3 (Millipore Corporation) was performed by using a Chromatin Immunoprecipitation Assay Kit (ChIP) (Millipore Corporation). Purified DNA from immunoprecipitates and input DNA were analyzed by quantitative PCR with primers for the mouse LINE1 and SINE B2 repetitive sequences. The results were normalized to the amount of input DNA and presented as fold change for each DNA in liver of mice from experimental groups relative to control mice.

Statistical analyses

Results are presented as mean ± S.D. Data were analyzed by one-way analysis of variance with pair-wise comparisons being made by the Student-Newman-Keuls method. When necessary, the data were natural log transformed before conducting analysis to maintain a more equal variance or normal data distribution. *P*-values < 0.05 were considered significant.

Results

Incidence of hepatic preneoplastic and neoplastic lesions

The mouse model of liver tumorigenesis in fibrotic liver was detailed in Uehara *et al*[11]. Both neoplastic (adenomas and carcinomas) and pre-neoplastic (foci) lesions were found in mice treated with DEN+olive oil, PBS+CCl$_4$, or DEN+CCl$_4$. Specifically, in the DEN+olive oil-treated group, foci were observed in 95% (19/20) of the mice and adenomas in 10% (2/20). In the PBS+CCl$_4$-treated group, the percentage of mice with foci, adenomas and carcinomas was 12.5% (1/8), 12.5% (1/8) and 25% (2/8), respectively. In the DEN+CCl$_4$-treated group, the percentage of mice with foci, adenomas and carcinomas was 31% (8/26), 100% (26/26)

and 50% (13/26), respectively. Hepatocellular adenomas displayed proliferation of relatively uniform hepatocytes accompanied with a loss of normal lobular architecture and compression of the surrounding parenchyma, while the histopathological features of hepatocellular carcinomas consisted of a broad trabecular growth pattern of atypical hepatocytes with hemorrhaging and ischemic necrosis in the center of the tumors (Supplementary Figure 1). Avascular and stromal invasions were also occasionally present in the hepatocellular carcinomas.

Mutational profiling of H-ras, Ctnnb1, and Hnf1α in liver tumors in mice treated with DEN and CCl₄

H-ras, Hnf1α, and Ctnnb1 genes were examined for the presence of mutations. Codon 61 of the *H-ras* gene has been identified as a hot spot for point mutations in both spontaneous and chemically-induced mouse hepatic tumors[21]. Although mutations in *HRAS* in human HCC are not common, overexpression of members of the RAS oncogene family, including *HRAS,* has been reported[22]. Mutations in *Ctnnb1* are a commonly observed event in hepatocarcinogenesis, most often found in the mouse exon 2 and in the corresponding human exon 3[23]. Human hepatocellular adenomas often contain inactivating mutations of the *HNF1A* gene[24]; these mutations have been suggested to be a result of exposure to genotoxic agents[25].

Mutations in codon 61 of *H-ras* were rare (1/24, 8.3%) in mice treated with DEN+CCl₄. One animal (with 13 adenomas and no carcinomas in its liver) harbored *H-ras* mutations, and the mutations were observed in both tumors and matched adjacent non-tumor liver tissue. Interestingly, the mutation was different in the tumor tissue (A→T transversion at the second base) and the adjacent non-tumor tissue (C→A transversion at the first base). No significant differences in gene expression of *H-ras* were observed between vehicle control, tumor, and adjacent non-tumor liver tissues of DEN+CCl₄-treated mice (Supplementary Figure 2).

Mutations in exon 2 of *Ctnnb1* were not observed in either tumor or adjacent non-tumor liver tissue of mice treated with DEN+CCl₄. Consistent with this finding, there were no differences in protein expression or cellular localization of CTNNB1 by immunohistochemistry (Figure 1, left panels). Tumors and adjacent normal tissues from mice treated with DEN+olive oil, PBS+CCl₄, or DEN+CCl₄ were negative for GLUL (Figure 1, middle panels), confirming the absence of an activating mutation in *Ctnnb1*. Because adenomas were found in all treatment groups, fatty acid binding protein (L-FABP1), a downstream target of *Hnf1α*, was used as a marker for inactivating mutations in *Hnf1α*. Liver tissues from animals in all groups were uniformly stained for FABP1, indicating a normal functioning *Hnf1α* (Figure 1, right panels).

DNA methylation changes in liver tumors in mice treated with DEN and CCl₄

It is well-established that both mouse and human liver tumors exhibit substantial alterations in DNA methylation[26]. Hence, the status of genomic and gene-specific DNA methylation in liver tumors induced by DEN+CCl₄ treatment was examined. Figure 2A shows that DNA was markedly hypomethylated both globally and within LINE1 and SINE B2 repetitive

elements in tumors as compared to liver tissue from vehicle control mice. It is well-established that the extent of methylation of these repetitive elements strongly corresponds to the level of global DNA methylation[27].

Figure 2B shows that five tumor suppressor genes, *Cdkn2a*, *Mgmt*, *Socs1*, *Cdh1*, and *Riz1,* all known to be commonly epigenetically altered in HCC[26,28], were heavily methylated in liver tumors in DEN+CCl$_4$-treated mice, while these genes were largely unmethylated in the livers of control mice. One of the main cancer-associated epigenetic features is the silencing of aberrantly methylated genes[29]. Hence, the expression of these five genes was also evaluated. Figure 2C shows that among the analyzed genes, only the expression of *Riz1* and *Mgmt* was significantly decreased in association with promoter hypermethylation in liver tumors from mice treated with DEN+CCl$_4$. Changes in the expression of *Cdkn2a*, *Socs1*, and *Cdh1* did not exhibit such an association with the incidence of promoter methylation.

DNA methylation changes in fibrosis-associated mouse hepatocarcinogenesis

To determine the role of epigenetic alterations in the progression of mouse fibrosis-associated liver carcinogenesis, the status of genomic DNA methylation in non-tumor liver tissues was assessed. Figure 3A shows that DNA isolated from non-tumor liver tissue of mice treated either with PBS+CCl$_4$ or with DEN+CCl$_4$ was also markedly hypomethylated. Interestingly, the extent of methylation of global DNA as well as of LINE1 and SINE B2 repetitive DNA sequences in non-tumorous liver tissue from mice treated with PBS+CCl$_4$ or with DEN+CCl$_4$ was similar to that of liver tumors in mice treated with DEN+CCl$_4$ (Figure 3A). In contrast to the results obtained in mice treated with PBS+CCl$_4$ or DEN+CCl$_4$, the level of DNA methylation in the livers of mice treated with DEN+olive oil was not different from that of control mice.

To further evaluate the role of DNA methylation changes in fibrosis-associated mouse liver carcinogenesis, promoter methylation and expression of *Cdkn2a*, *Mgmt*, *Socs1*, *Cdh1*, and *Riz1* in non-tumor liver tissue were examined. Figure 3B shows that these tumor suppressor genes were methylated in the non-tumorous liver samples in DEN+CCl$_4$-treated mice, while they were largely unmethylated in the livers of control mice. However, the extent of *Mgmt*, *Socs1*, and *Riz1* methylation in non-tumor tissue was noticeably lower than in liver tumors. Interestingly, in PBS+CCl$_4$-treated mice, *Cdkn2a*, *Socs1*, *Cdh1*, and *Riz1* were methylated in 40% of the non-tumorous liver samples. In DEN+olive oil-treated mice, only *Socs1* was methylated in 40% of adjacent non-tumor liver samples.

Figure 3C shows that among all of the analyzed genes, only the expression of *Mgmt* and *Riz1* was significantly decreased in association with promoter hypermethylation in the non-tumor liver tissues of mice treated with DEN+CCl$_4$. Changes in the expression of *Cdkn2a*, *Socs1*, and *Cdh1* did not exhibit such an association with the incidence of promoter methylation. The expression of these genes was similar in non-tumor and tumor tissue from the livers of mice treated with DEN+CCl$_4$ (Figures 2C and 3C).

Histone methylation during fibrosis-associated mouse hepatocarcinogenesis

It has been previously reported that a reduction in trimethylation of H3K9, H3K27, and H4K20 affects genomic stability[30,31], and is an event observed in various human cancers.

Figure 4 shows a large decrease in H3K9 trimethylation in the livers of mice treated with DEN+CCl$_4$. The levels of H3K9 trimethylation in this experimental group were decreased by 58% compared with control mice. In contrast to the H3K9 methylation changes, the levels of H3K27 and H4K20 were decreased only slightly in the livers of DEN+olive oil-, PBS+CCl$_4$-, and DEN+CCl$_4$-treated mice.

Expression of chromatin-modifying genes during fibrosis-associated hepatocarcinogenesis

To determine the underlying mechanisms of these epigenetic aberrations, the expression of chromatin modifying genes involved in the proper maintenance of DNA and histone methylation was assessed by qRT-PCR. The most noticeable changes that occurred during fibrosis-associated carcinogenesis were a distinct up-regulation of the maintenance DNA methyltransferase 1 (*Dnmt1*) and *de novo* DNA methyltransferase 3a (*Dnmt3a*) genes in the livers of mice treated with PBS+CCl$_4$ and DEN+CCl$_4$. Down-regulation of histone lysine (K)-specific demethylase (*Kdm4a* and *Kdm4b*) genes was observed in liver tissue of mice treated with DEN+CCl$_4$ (Figure 5).

Histone H3K9 trimethylation and expression of LINE1 and SINE B2

To investigate whether or not epigenetic changes observed in this study may be mechanistically related to the progression of fibrosis-associated carcinogenesis, the status of H3K9 methylation at LINE1 and SINE B2 repetitive sequences and their expression were analyzed. Figure 6A shows a substantial decrease of H3K9 trimethylation (H3K9me3) at LINE1 and SINE B2 repetitive sequences in liver tissue of mice treated with DEN+CCl$_4$. The reduction of LINE1 and SINE B2 H3K9me3 was accompanied by a marked increase in the expression of LINE1 and, especially, SINE B2 elements (Figure 6B).

Expression of progenitor markers in liver tumors

In this mouse model of hepatocarcinogenesis, markers of oncofetal liver transformation and cancer stem cells were found to be significantly associated with tumor incidence; whereas inflammation, fibrogenesis, oxidative stress, proliferation and apoptosis were not indicative of increased tumorigenesis[11]. Supplementary Figure 3 shows that the expression of alpha-fetoprotein (*Afp*) was significantly up-regulated in tumor tissues of mice treated with DEN+CCl$_4$ when compared to control animals, a pattern similar to that reported for the non-tumor tissues from the same treatment group[11]. In contrast, epithelial cell adhesion molecule (*Epcam*) was down-regulated in tumor tissues when compared to control mice, although the change was not statistically significant. Interestingly, *Epcam* was significantly up-regulated in non-tumor tissues of the DEN+CCltretaed mice when compared to all other treatment groups in the original study[11]. These findings corresponded to the hypothesis that this mitogenic signaling molecule is involved in tumor promotion and progression, but is less active in late stage tumors.

Discussion

Hepatocellular carcinogenesis is a complex process that is the consequence of multiple molecular events that lead to the initiation, promotion, and progression of tumor cells[3,4,6,7].

Recent reviews have emphasized the importance of a distinct set of events that are required for carcinogenesis[32,33]. Specifically, distinct cellular capabilities that enable tumorigenesis, or "hallmarks of cancer," are increasingly recognized as essential processes in carcinogenesis[32]. Mutations in cancer-related genes result in aberrant cellular functions, which can be characterized as specific hallmark capabilities. While genetic alterations have long been known to cause the development and progression of tumors, epigenetic events have emerged more recently as potentially equally important events in the process of hepatocarcinogenesis[13,26].

To better understand the role of genetic and epigenetic alterations in liver tumor development, the mutational profile, expression, and epigenetic alterations of several cancer-related genes commonly involved in hepatocarcinogenesis were evaluated using a mouse model of fibrosis-associated hepatocellular carcinoma. Additionally, epigenetic events associated with genomic instability, such as global DNA hypomethylation and histone demethylation, were investigated.

Common mutations do not play a primary role in fibrosis-associated HCC in murine liver tumors

We evaluated the mutational profile of genes commonly implicated in hepatocarcinogenesis of both mice and humans. Alterations in the Wnt/β-catenin pathway are frequently involved in both human and mouse HCC, with *CTNNB1* reported to be the most commonly activated oncogene in human HCC[23]. Activating mutations in *CTNNB1* lead to constitutive activation of the CTNNB1 protein and subsequent up-regulation of Wnt signaling,[34] the consequence of which is stimulation of cell proliferation and inhibition of apoptosis[35]. Recent studies have reported contrasting results regarding the association of *CTNNB1* mutations and genomic instability in hepatic tumors[33,36]. However, a general consensus has been established that genomic instability is an enabling characteristic in hepatic and other cancers[32]. In our study, while epigenetic alterations that are associated with genomic instability were observed, mutations in the hot-spot region (mouse exon 2) of *Ctnnb1* were absent. These findings suggest that causative mechanisms of genomic instability may precede *Ctnnb1* mutation, and support the theory that genomic instability and *Ctnnb1* mutation are distinct mechanisms of hepatocarcingenesis[6,35].

Characterizing the frequency and type of mutations in tumor-related genes is informative in the determination of tumor induction and progression, especially in laboratory animals, because the basis of carcinogenic effects may be due to either chemical induction or spontaneous occurrence[37]. For example, single base substitution mutations at codon 61 of *H-ras* are one of the most commonly seen mutations in spontaneously occurring liver tumors in B6C3F1 mice[21]. Mutations at this hotspot are also seen in chemically-induced neoplasms in mice, although there is evidence that the type and frequency of mutations can be chemical- and dose-dependent, with a lower incidence of codon 61 mutations associated with increasing doses of multiple chemicals[14, 38], including DEN[21]. Indeed, the low frequency of *H-ras* mutations in our study suggests that CCl_4 and DEN together preferentially promote cells through mechanisms independent of *H-ras* mutation.

Epigenetic alterations are an early and important event in fibrosis-associated mouse hepatocarcinogenesis

Epigenetic changes such as DNA hypomethylation at the global level and within repetitive sequences, promoter hypermethylation of tumor suppressor genes, and altered histone lysine methylation are common characteristics in many, if not all, types of cancer[29,39,40]. In our study, the extent of global DNA hypomethylation was significantly greater in the livers of PBS+CCl_4- and DEN+CCl_4-treated mice than in DEN+olive oil-treated or control mice. Similarly, demethylation of LINE1 and SINE 2B sequences was found in PBS+CCl_4- and DEN+CCl_4-treated mice, although the extent of LINE1 demethylation in the livers of PBS+CCl_4-treated mice was not statistically significant. Furthermore, we observed a significant decrease in global H3K9me3 in the non-tumor liver tissue in mice treated with DEN+CCl_4. Additionally, the degree of DNA methylation and H3K9me3 was significantly decreased in LINE1 and SINE B2 sequences in the livers of DEN+CCl_4-treated mice, and the expression of LINE1 and SINE B2 was significantly increased. These interspersed repetitive sequences, which represent nearly half of the human genome and approximately one-third of the mouse genome,[41] play an important role in the development of several human cancers[42].

Notably, the PBS+CCl_4- and DEN+CCl_4-treated mice had a higher incidence of liver tumors (37.5% and 100%, respectively) than mice treated with DEN+olive oil (10%), as reported by Uehara, *et al.* in 2013. While the incidence of liver preneoplastic foci was nearly 100% in DEN+olive oil-treated mice, few of these lesions (2/20) progressed to adenomas. This suggests that the significant increase in tumor incidence in livers of mice treated with DEN+CCl_4, as compared to the other treatment groups, may be attributed to a significant hypomethylation of genomic DNA, LINE1 and SINE B2 repetitive sequences, and the decrease in global, LINE1 and SINE B2 H3K9me3. Importantly, these epigenetic alterations appeared to precede genetic aberrations commonly seen in mouse and human HCC.

RIZ1, a member of a histone/protein methyltransferase superfamily, is associated with tumor suppression function[43] and is frequently inactivated in HCC[44,45]. It has been demonstrated that the histone methyltransferase function of RIZ1 is an important constituent of its tumor suppressor activity[46]. Our results indicate that inhibition of *Riz1* by promoter hypermethylation is associated with a substantial decrease in H3K9me3, which may be the result of lost RIZ1 histone methyltransferase function. A significant decrease in *Riz1* expression was found in both tumor and non-tumor liver tissues of mice treated with DEN+CCl_4, as well as in the livers of mice treated with PBS+CCl_4 alone. Because mice in these treatment groups had a higher tumor incidence than DEN+olive oil-treated mice, we can infer that *Riz1* plays a major role in tumorigenesis in our study. The level of H3K9me3 was significantly decreased only in mice treated with DEN+CCl_4, suggesting a closely link between H3K9me3 and tumor cell promotion and/or progression. This also suggests that mechanisms additional to the loss of *Riz1* histone methyltransferase function play a role in H3K9 methylation.

RIZ1 promoter hypermethylation has been observed in both early and advanced stages of human HCC, with a higher incidence in early stage tumors[47]. This supports the potential tumor-promoting role of *Riz1* promoter hypermethylation found in the present study, and also confers the mechanistic human relevance of this mouse HCC model. Additionally,

RIZ1 methylation has been associated with a shorter disease-free survival time in human HCC patients[45]. *RIZ1* hypermethylation has been found to be prevalent in various human cancer types, particularly breast and liver, two types in which mutations in the gene have not been found[43]. These findings indicate that this gene is likely preferably silenced by methylation as opposed to by mutation[43]. Furthermore, there are no known cis-acting regulators of *RIZ1* expression[48], which supports the conclusion that methylation is the cause for the down-regulation of the gene in many cancers.

Not all epigenetic events may be involved in carcinogenesis

We found that certain early epigenetic events appear to occur without effect on gene expression or be associated with tumor promotion. For example, although promoter hypermethylation was observed in four (*Cdkn2a, Mgmt, Socs1,* and *Cdh1*) of the cancer-related genes examined in this study, the expression of these genes was not down-regulated. This is contrary to the well accepted mechanistic link between promoter hypermethylation and gene silencing[27]. In fact, *Cdkn2a* and *Cdh1* were up-regulated in the livers of PBS $+CCl_4$- and DEN+CCl_4-treated mice a finding consistent with the hypothesis that not all DNA methylation changes, similar to genetic mutations, are equally important in the process of carcinogenesis, and that some DNA methylation changes may be purely passenger events[49].

Conclusions

Our results indicate that epigenetic alterations are an essential and early event in hepatocellular carcinogenesis, especially under conditions of liver fibrosis. Based on previous reports, genomic instability is likely a consequence of loss of DNA methylation globally and within repetitive elements and decreased H3K9 methylation, all of which were associated with increased tumor incidence in the present study. Furthermore, loss of *Riz1* expression by promoter hypermethylation was associated with increased tumor incidence, and therefore the tumor suppressor function of this gene is likely linked to its inherent histone methyltransferase activity. The epigenetic alterations observed in fibrosis-associated liver tumors in this mouse model indicate important features involved in the development of human liver tumors that arise from fibrosis and cirrhosis, a common progression according to human clinico-pathological evidence. Additionally, these epigenetic alterations may confer a heightened risk for genomic instability, although additional studies are required to confirm such an association. Our results indicate that *H-ras, Hnf1*α, and *Ctnnb1* mutations are not involved in the initiation or promotion of the hepatic tumors in this study, and that epigenetic changes preceded or occurred independently of traditionally observed genetic alterations in mouse HCC. While common HCC-related mutations were not detected in our study, the epigenetic alterations observed may contribute to genomic instability and thus represent one of the proposed hallmarks of cancer[32].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Novelty and impact statement

This report demonstrates that epigenetic modifications, rather than mutations in known cancer-related genes, are essential and early events in mouse hepatocellular carcinogenesis that play a prominent role in the increased incidence of liver tumors in fibrosis-associated liver cancer. The epigenetic alterations observed in a mouse model of fibrosis-associated liver tumors may indicate important features of tumor development in human liver tumors that arise from fibrosis and cirrhosis.
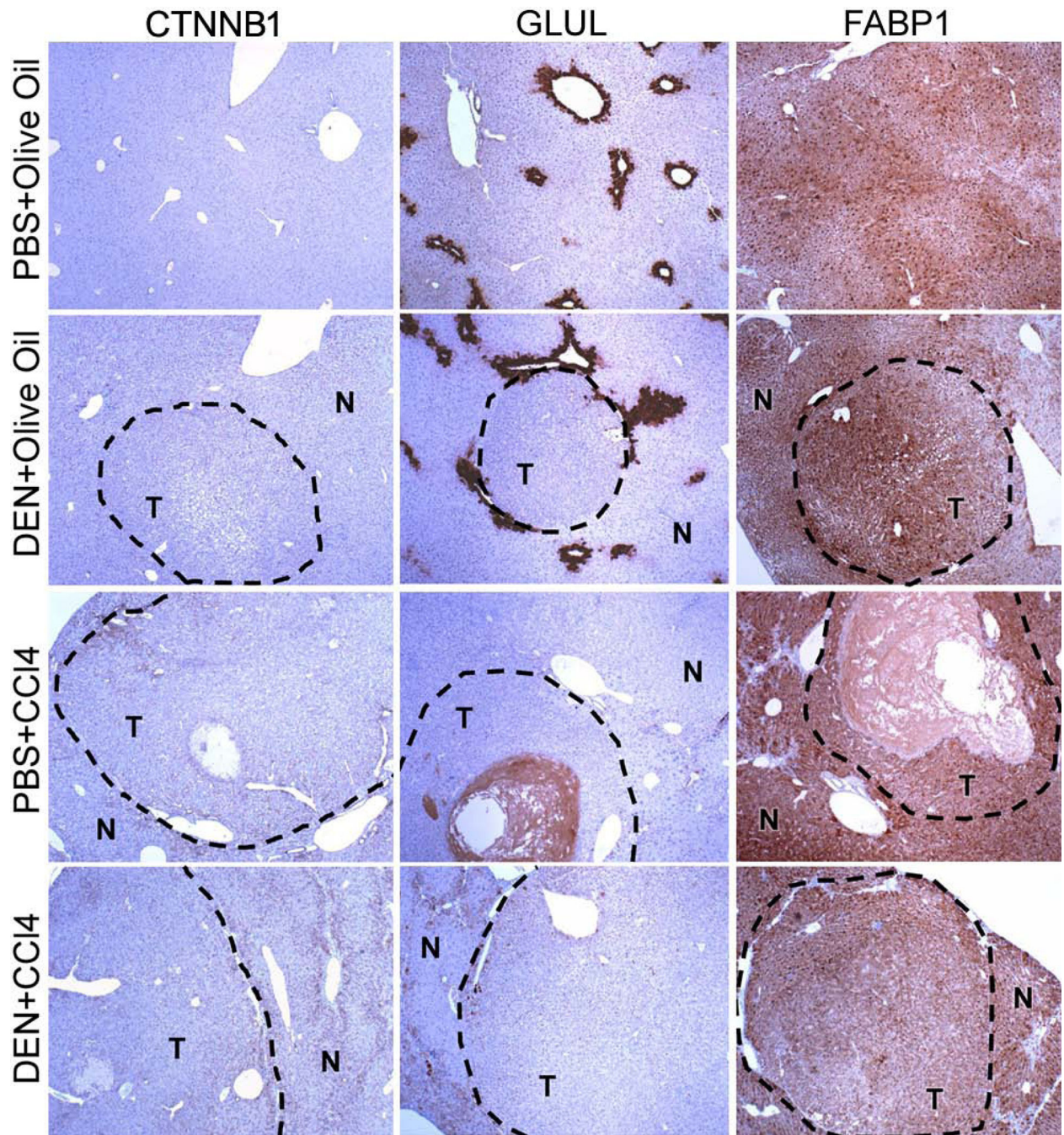
Figure 1.
Immunostaining of CTNNB1, GLUL, and FABP1 in formalin-fixed paraffin-embedded sections of liver tumor (T) and adjacent non-tumor (N) liver tissue. *Left panel:* Liver tumors (pictured in all groups except vehicle control) lack immunoreactivity or nuclear accumulation of CTNNB1. Minimal cytoplasmic accumulation is present in centrilobular hepatocytes in PBS+CCl$_4$-treated mice, primarily in non-tumor fibrotic areas and likely artifact from degeneration. *Middle panel:* Liver tumors (pictured in all groups except vehicle control) lack immunoreactivity to GLUL. Positive immunoreactivity is restricted to

pericentral hepatocytes in non-tumor tissue. Pericentral immunoreactivity is weak in PBS +CCl$_4$-treated mice, likely due to degeneration and loss of these hepatocytes. The positive staining in the PBS+CCl$_4$ representative image is artifact due to necrosis. *Right panel:* Uniform positive immunoreactivity is present in both tumor (pictured in all groups except vehicle control) and adjacent non-tumor tissue. Original magnification: 40×.
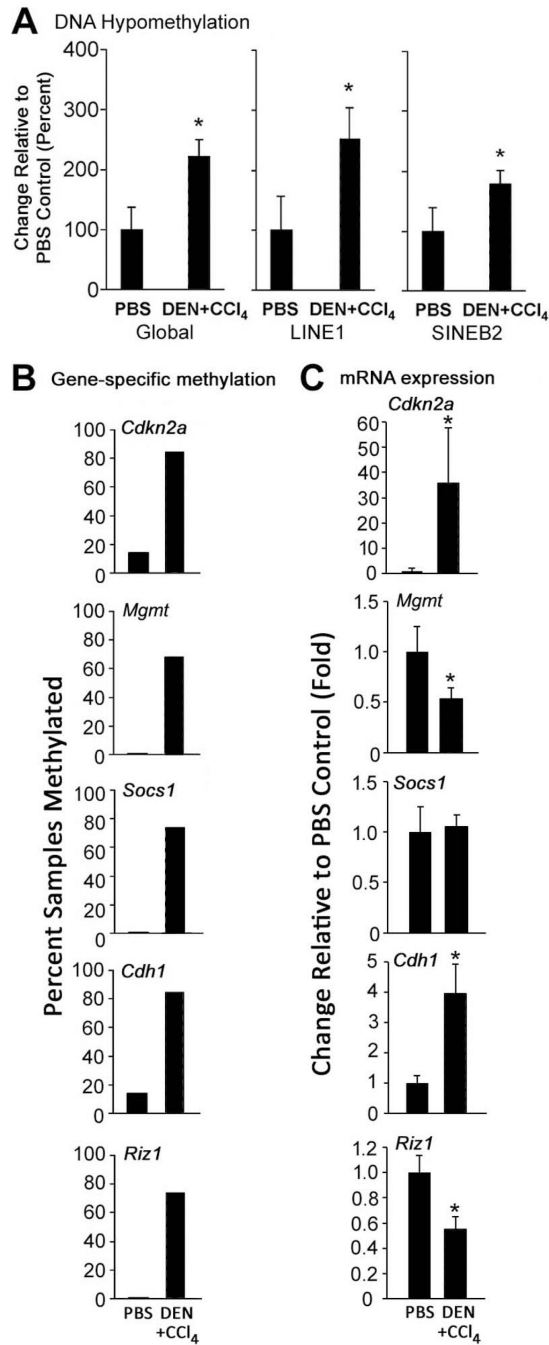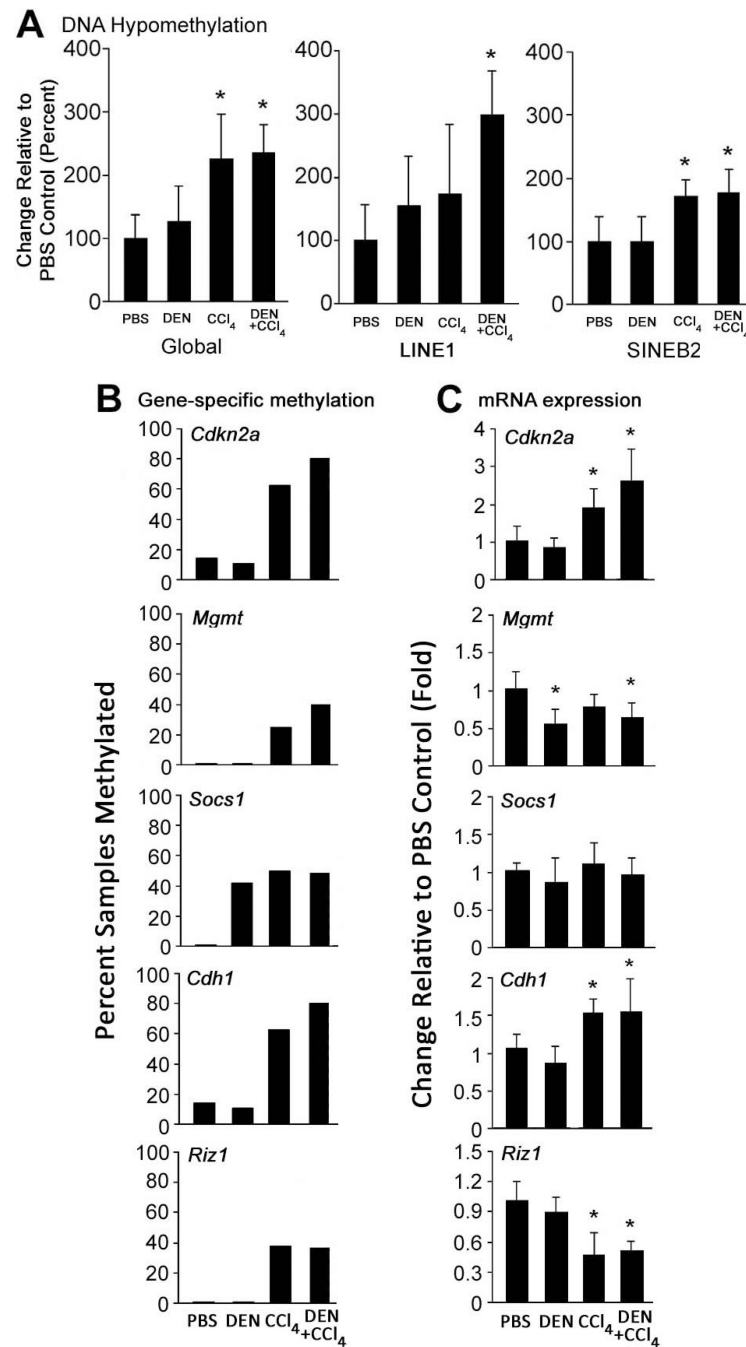
Figure 2.
DNA methylation and gene expression changes in liver tumors. **(A)** Genomic, LINE1, and SINE B2 methylation. The results are presented as an average percent change in the degree of DNA hypomethylation in liver tumors of mice treated DEN+CCl$_4$ relative to that in the PBS (vehicle control) group, which were assigned a value of 100%. **(B)** Extent of *Cdkn2a, Mgmt, Socs1, Cdh1* and *Riz1* promoter methylation. The Y-axis represents the percentage of samples that had methylation in the promoter region of the described gene. **(C)** The expression of *Cdkn2a, Mgmt, Socs1, Cdh,1* and *Riz1* was determined by qRT-PCR as

detailed in "Materials and Methods." The results are presented as an average fold change in the expression of each gene in liver tumors of mice treated DEN+CCl$_4$ relative to expression in liver tissues of the control group, which were assigned a value of 1.

Values shown are mean±S.D., asterisks (*) denote a significant (p<0.05) difference from control mice.

Figure 3.
DNA methylation and gene expression changes in non-tumor liver tissues during fibrosis-associated hepatocarcinogenesis in mice. **(A)** Genomic, LINE1, and SINE B2 methylation. The results are presented as an average percent change in the degree of DNA hypomethylation in non-tumorous liver tissues of mice from experimental groups relative to that in control mice, which was assigned a value of 100%. **(B)** Extent of *Cdkn2a, Mgmt, Socs1, Cdh1* and *Riz1* promoter methylation. The Y-axis represents the percentage of samples that had methylation in the promoter region of the described gene. **(C)** The

expression of *Cdkn2a, Mgmt, Socs1, Cdh1,* and *Riz1* genes. The results are presented as an average fold change in the expression of each gene in the livers of mice from experimental groups relative to that in control mice. Values shown are mean±S.D., asterisks (*) denote a significant (p<0.05) difference from control mice.
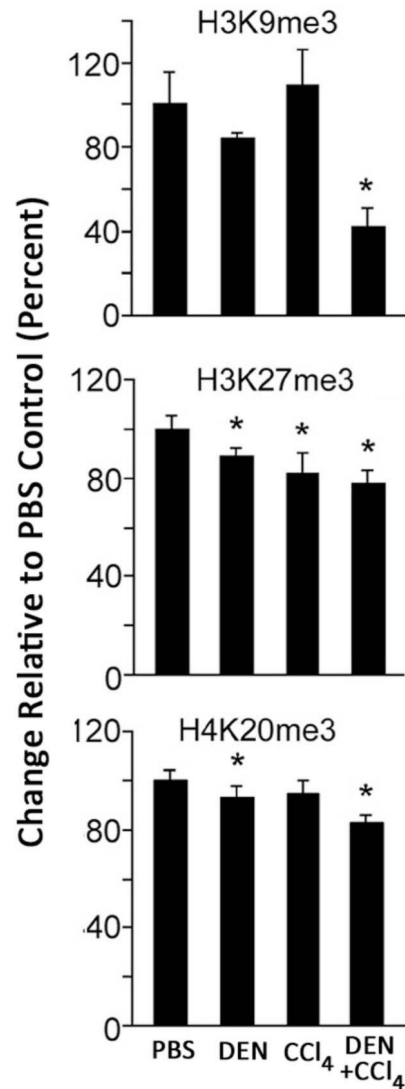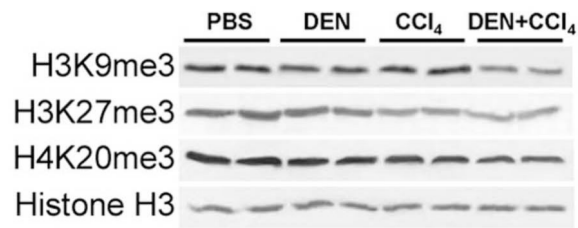
Figure 4.
H3K9, H3K279, and H4K20 trimethylation in liver tissues during fibrosis-associated liver carcinogenesis in mice. Densitometry analysis of the immunostaining results is shown as percent change in histone modification level in the each experimental group relative to the corresponding values in control mice. Values shown are mean±S.D., asterisks (*) denote a significant (p<0.05) difference from control mice.
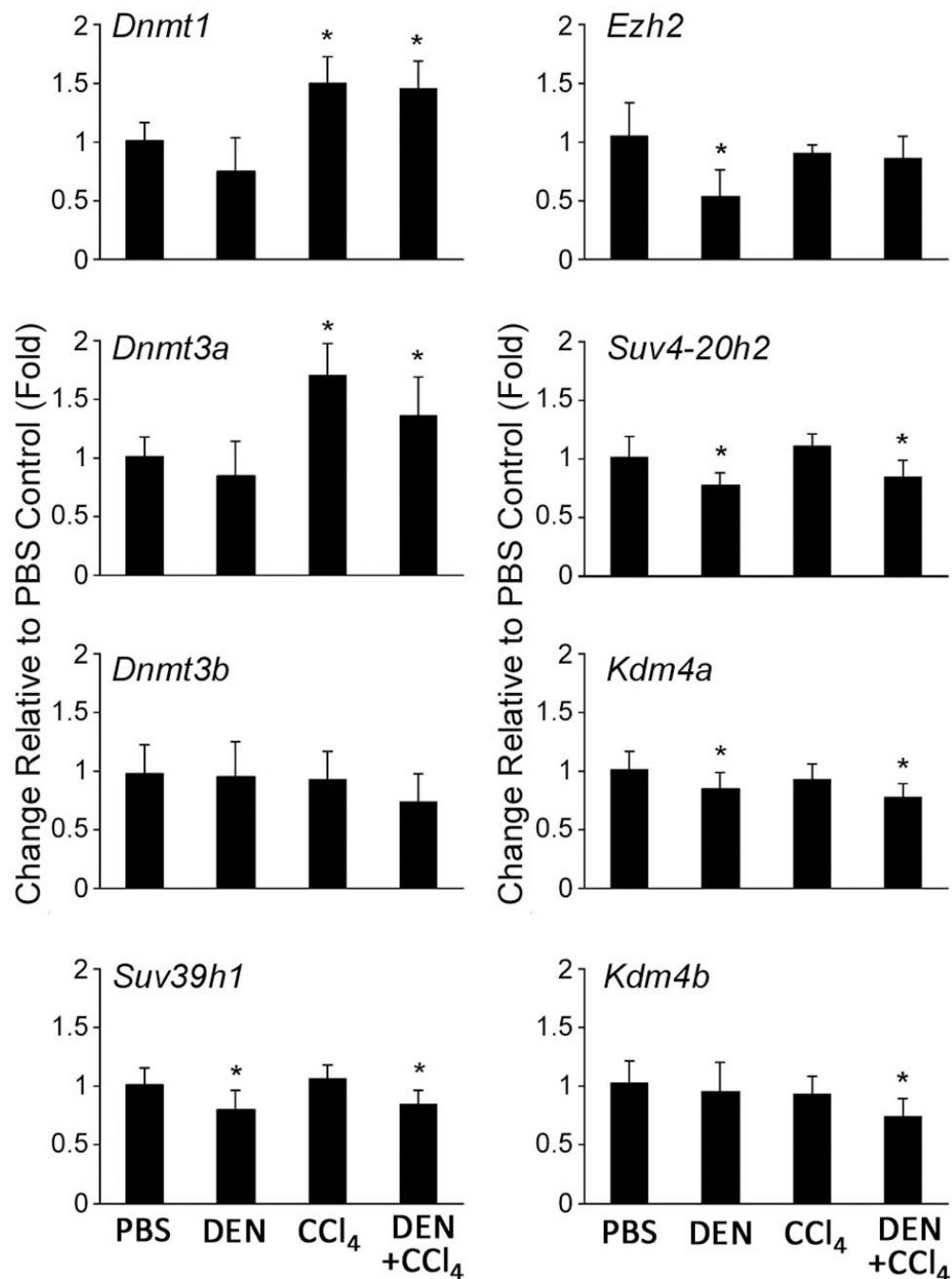
Figure 5.
Expression of chromatin-modifying genes in the liver during fibrosis-associated hepatocarcinogenesis in mice. The expression of *Dnmt1, Dnmt3a, Dnmt3b, Suv39h1, Ezh2, Suv4-30h2, Kdm4a,* and *Kdm4b* genes was determined by qRT-PCR. The results are presented as an average fold change in the expression of each gene in the livers of mice from each experimental group relative to that in control mice, which were assigned a value 1. Values shown are mean±S.D., asterisks (*) denote a significant ($p < 0.05$) difference from control mice.
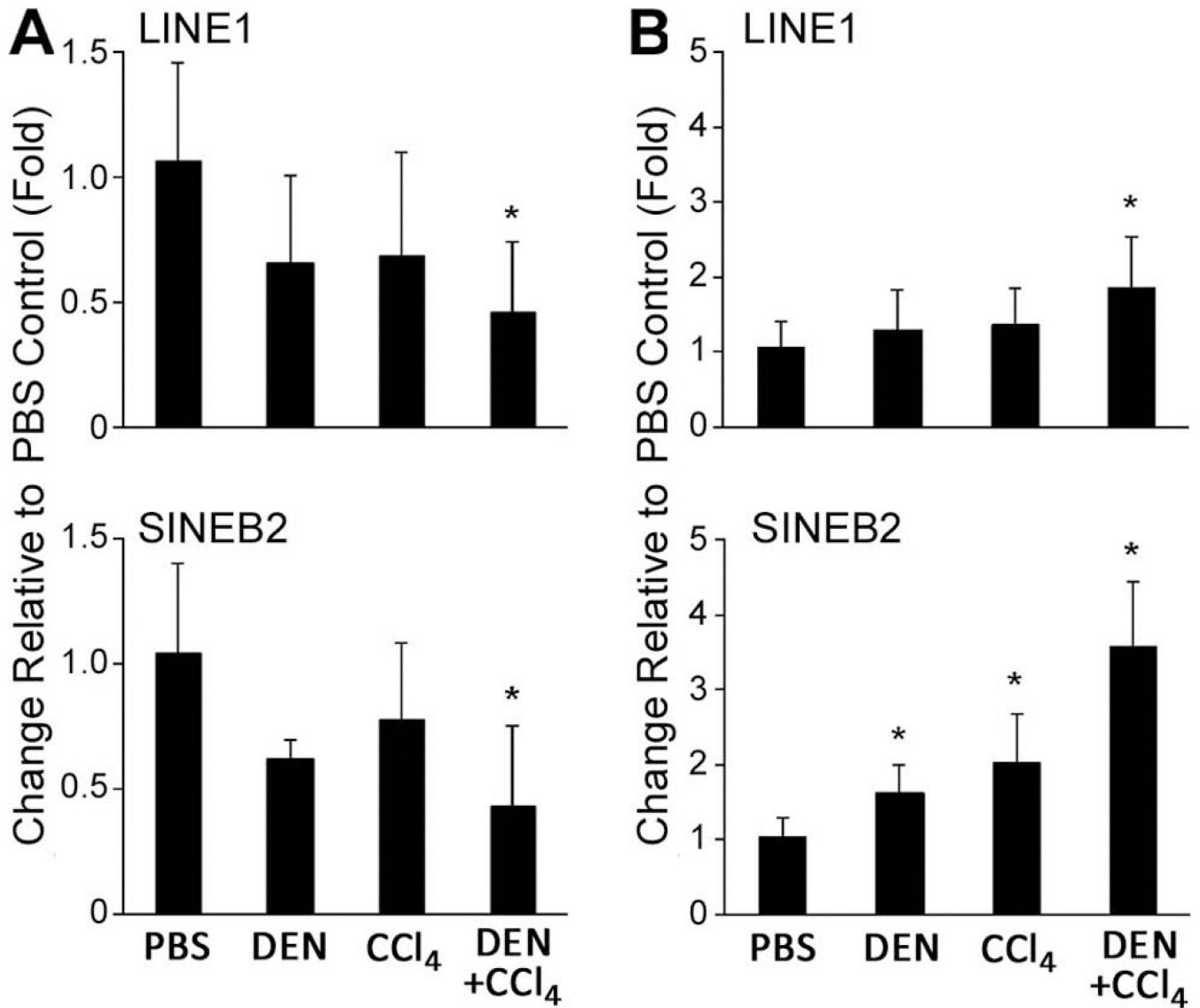
Figure 6.
Level of H3K9me3 at LINE1 and SINE B2 repetitive sequences and expression of LINE1
and SINE B2 in the liver during fibrosis-associated liver carcinogenesis in mice. **(A)** The
level of H3K9me3 at LINE1 and SINE B2 determined by a ChIP assay as described in
"Materials and Methods." The data are presented as fold change in liver of mice from
experimental groups relative to control mice after normalization to input DNA. **(B)**
Expression of LINE1 and SINE B2 elements. The results are presented as an average fold
change in the expression of LINE1 and SINE B2 in the livers of mice from experimental
groups relative to that in control mice, which were assigned a value of 1. Values shown are
mean±S.D., asterisks (*) denote a significant (p<0.05) difference from control mice.