UUHigher Susceptibility to Aflatoxin B_1-related Hepatocellular Carcinoma in Glycine N-Methyltransferase Knockout Mice

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Significance

Glycine N-methyltransferase (GNMT) is an enzyme with multiple functions. It expresses abundantly in liver, but is down-regulated in human hepatocellular carcinoma (HCC). Previously we reported that by knocking out the gene expression of Gnmt in mice, 50% male mice and all female animals developed HCC spontaneously. We report here that the absence of Gnmt expression could accelerate AFB_1-induced liver tumorigenesis. According to our results, 62.5% (5/8) of male and 80% (4/5) of female Gnmt-/- mice developed HCC by 14 months of age, approximately 6 months earlier than AFB_1-treated wild-type mice. Results from microarray and real-time PCR analyses indicate that five genes related to detoxification pathway were down-regulated in AFB_1-treated Gnmt-/- mice. Therefore, homeostasis of GNMT gene expression is very important for maintenance of detoxification capacity of an animal.

Abstract

In both humans and rodents, males are known to be more susceptible than females to hepatocarcinogenesis. We have previously reported that glycine N-methyltransferase (GNMT) interacts with aflatoxin B_1 (AFB_1) and reduces both AFB_1-DNA adduct formation and hepatocellular carcinoma (HCC) in mice. We also reported that 50% of the males and 100% of the females in a small group of Gnmt null (Gnmt-/-) mice developed HCC, with first dysplastic hepatocellular nodules detected at mean ages of 17 and 16.5 months, respectively. In this study we tested our hypothesis that male and female Gnmt-/- mice are susceptible to AFB_1 carcinogenesis, and that the absence of Gnmt expression may accelerate AFB_1-induced liver tumorigenesis. We inoculated Gnmt-/- and wild-type mice intraperitoneally with AFB_1 at 7 days and 9 weeks of age and periodically examined them using ultrasound. Dysplastic hepatocellular nodules were detected in 6 of 8 males and 5 of 5 females at 12.7 and 12 months of ages, respectively. Dysplastic hepatocellular nodules from 5/8 (62.5%) male and 4/5 (80%) female Gnmt-/- mice were diagnosed as having HCC, approximately 6 months earlier than AFB_1-treated wild-type mice. Results from microarray and real-time PCR analyses indicate that five detoxification pathway-related genes were down-regulated in AFB_1-treated Gnmt-/- mice: Cyp1a2, Cyp3a44, Cyp2d22, Gsta4 and Abca8a. In summary, we observed overall higher

susceptibility to AFB_1-related HCC in Gnmt-/- mice, further evidence that GNMT over-expression is an important contributing factor to liver cancer resistance.

Introduction

Human hepatocarcinogenesis is a multistage process with multifactorial etiology, including genetic and environmental interactions. The risk factors associated with HCC include chronic infection with the hepatitis B or C virus, exposure to dietary aflatoxin B1 (AFB_1) on moldy corn and vegetables, and that simultaneous infection with hepatitis B virus and ingestion of Aflatoxin B1 leads to a synergistic increase in liver carcinogenesis and HCC.[1] Two unusual phenomena have been observed in the epidemiology of human hepatocellular carcinoma (HCC): (a) high morbidity in sub-Saharan Africa and eastern Asia, implying a large prevalence of HBV and the contamination of foodstuffs with AFB_1; and (b) regardless of region, HCC is more prevalent in males, with reported male-to-female ratios in most countries ranging from 2:1 to 6:1.[1-3]

Aflatoxin ingestion has been identified as a major risk factor for HCC development in Africa and Asia.[4-6] Aflatoxin B_1 (AFB_1) epoxide binds covalently to DNA and induces G-to-T transversions at the third base in codon 249 of the p53 gene [7, 8]. Male mice have been shown to be more susceptible than female mice to AFB_1-induced liver tumor formation,[9, 10] and multiple proteins are known to be capable of binding with AFB_1 in rat liver cytosols.[11]

We recently reported that glycine N-methyltransferase (GNMT) can bind with

AFB_1 and inhibits DNA adduct formation.[12] We also used AFB_1 to challenge GNMT transgenic mice intraperitoneally. After 11 months we found that neither male nor female GNMT transgenic mice had developed HCC, unlike the 67% of male wild-type mice that did develop the disease.[12] Results from experiments using recombinant adenoviruses carrying GNMT cDNA (Ad-GNMT) provide further evidence that the GNMT-related inhibition of AFB_1-DNA adduct formation is dose-dependent. Results from HPLC analyses of AFB_1 metabolites in the culture supernatant of cells exposed to AFB_1 indicate that aflatoxin M1 (AFM_1) levels in the GNMT group were significantly higher than in the control group, suggesting that the presence of GNMT can enhance the AFB_1 detoxification pathway.

GNMT, an enzyme with multiple functions, affects genetic stability by regulating the ratio of S-adenosylmethionine to S-adenosylhomocystine and by binding to folate.[13, 14] GNMT is expressed in human liver, pancreas, and prostate tissues.[15] Aida *et al*. (1997) found that GNMT expression regulated by growth hormone is much lower in the mouse liver tissues of males than in females at 6 weeks of age.[16] In 1998 we reported that GNMT expression was down-regulated in human HCC tissues and cell lines.[17] In addition, genotypic analyses of several human GNMT genetic polymorphisms indicate a loss of heterozygosity in 36-47% of genetic markers in HCC tissues.[18] After generating Gnmt null (Gnmt-/-) mice, we

demonstrated that they develop chronic hepatitis and glycogen storage disease (GSD) in the liver.[19] Results from pathological examinations indicate focal necrosis in male Gnmt-/- mice livers and degenerative changes in the intermediate zones of female Gnmt-/- mice livers.[19] In a related study, 3 of 6 male and 7 of 7 female Gnmt-/- mice developed HCC.[20] We therefore proposed that GNMT is a tumor suppressor gene, and that its gender-specific overexpression in female mice is a likely reason for the gender disparity in HCC susceptibility.

For the present study we hypothesized that Gnmt-/- mice are susceptible to AFB_1 carcinogenesis, and that Gnmt deficiency may accelerate AFB_1-induced liver tumorigenesis. According to our results, 62.5% (5/8) of male and 80% (4/5) of female mice developed HCC by 14 months of age, approximately 6 months earlier than AFB_1-treated wild-type mice. Data from microarrays and real-time PCR were used to compare signal transduction and detoxification pathways between wild-type and Gnmt-/- mice.

Materials and Methods

Animals.

The Gnmt-/- mice and wild-type mice used in this study were from the same litter and shared the same genetic background as 129/B6 (129sv X C57BL/6).[19] A male heterozygous Gnmt knockout (Gnmt +/-) mouse was intercrossed with a female Gnmt+/- mouse (both with 129/B6 genome backgrounds) to generate Gnmt-/-, Gnmt+/-, and wild-type mice. As described previously, we used PCR with specific primers to differentiate the three types.[19] Mice were housed in a pathogen-free animal facility under a standard 12 h light/12 h dark cycle with water and chow. A combination of ultrasound and magnetic resonance imaging (MRI) was used to monitor animals for evidence of liver tumorigenesis. Experimental protocols were approved by the Institutional Animal Care and Use Committee of National Yang-Ming University.

AFB_1 challenge.

AFB_1 (Sigma Co., St Louis, MO) was dissolved in tricaprylin (Sigma) at a concentration of 0.2 mg/mL. At 7 days of age, Gnmt-/- and wild-type mice from the same litter were intraperitoneally injected with 10 mg AFB_1/kg of body weight; a second treatment of 40 μg AFB_1 per mouse was given at 9 weeks of age. Solvent

control groups were treated with tricaprylin at 7 days and 9 weeks of age. At least 3 mice from each group were included in this study. This protocol is a modification of procedures described by Ghebranious and Sell.[9] Serum alanine aminotransferase (ALT) levels were analyzed using Fujifilm DRI-CHEM 3500s (Kanagawa, Japan).

Autopsy.

Mice were sacrificed for pathological examination at 3 and 6 months of age. For the hepatocarcinogenesis study, complete autopsies were performed on mice with nodules >0.5 cm. Liver lobes were separated and photographed. Each grossly visible nodule or tumor was measured and counted, and data were recorded for liver lobes. Lobes were divided into two groups, one fixed in buffered formalin for later hematoxylin and eosin (H&E) and reticulin staining,[20] and the other stored in liquid nitrogen for DNA, RNA, and protein analyses. H&E and reticulin staining procedures are described in Young and Heath.[21]

Immunohistochemical Staining (IHC).

Slides were incubated with mouse anti-human Ki67 antibody at 4°C overnight (1:100 dilution in blocking buffer) (BD Biosciences, San Jose, CA). After three washings with PBS, we applied a streptavidin-biotin-peroxidase complex according to the

manufacturer's instructions (Lab Vision Corporation, Fremont, CA). Reactions were treated with 3,3' diaminobenzidine tetrahydrochloride (DAB) (Dako Corporation, Copenhagen) and counterstained with hematoxylin for 5 minutes. Reactions were stopped with 10mM Tris-HCl (pH8.0). Slides were dehydrated using an ascending ethanol series and mounted in Entellan New rapid embedding agent (MERCK, Darmstadt, Germany). Ki-67 staining was quantitated by the average of positive staining cell numbers in five different areas.

Microarray analysis.

Mice sacrificed at 3 months of age were used to study the gene expression profiles by microarray analysis; data are available at http://www.ncbi.nlm.nih.gov/project/geo (GSE18218). At 11 weeks, both AFB_1-treated Gnmt-/- and wild-type mice were subjected to microarray analyses. Microarray data for untreated Gnmt-/- and wild-type mice are presented in Liao *et al*.;[20] these data were used for comparison with data from this study. RNA was prepared from pooled liver tissue; purification, hybridization, and washing were performed as described in Brazma *et al*. [22] and Lee *et al*.[23] Briefly, 40 μg of total RNA were reverse-transcribed into cDNA with Cy3 or Cy5 monoreactive fluorophors using a CyScribe First-Strand cDNA labeling kit (Amersham Biosciences, Piscataway, NJ) and Superscript II RT (Invitrogen Life

Technologies, Carlsbad, CA). A T7-(dT24) oligonucleotide was used as a primer. All cDNA samples were hybridized to Mouse Genome 430A 2.0 arrays (Affymetrix), with individual arrays representing approximately 14,000 well-characterized mouse genes. Data were normalized and averaged after deleting incorrect and skewed values. Up-regulation was defined as a ratio of Gnmt-/- to WT mice $\geqq 2$ and downregulation as a ratio $\leqq 0.5$. Hierarchical clustering was used to detect overall expression patterns collected from different groups using the DNA-Chip Analyzer (http://www.dChip.org/). Gene expression patterns in different pathways were analyzed using the Babelomics web tool (http://babelomics.bioinfo.cipf.es/cgi-bin/tools.cgi) and KEGG pathways database (http://www.genome.jp/kegg/pathway.htm).

Real-time analysis.

Real-time PCR was used to determine the gene expression levels of Cyp1a2, Cyp3a44, Cyp2d22, Gsta4 and Abca8a in Gnmt-/- and wild-type mice treated with either AFB_1 or solvent at 3 months of age. At least 3 mice in each group were used to perform real-time analyses. Complementary DNA was produced from hepatic RNA (5 μg). See Mato *et al*. for a detailed description of our real-time PCR analysis procedures.[24] Primer sequences are listed in Table I.

Statistical analysis.

We used Wilcoxon rank sum tests to determine differences in liver weight/total body weight ratios and serum ALT levels between Gnmt-/- and wild-type mice. Student's *t* tests were used to compare gene expression profiles of Gnmt-/- and wild-type mice. Data were analyzed using the SAS program version 9.0 (SAS Institute, Cary, NC) and *p* values were calculated from two-tailed statistical tests, with a α level of 0.05.

Results

Liver tumor formation in wild-type and Gnmt-/- mice treated with AFB_1.

As shown in Table II, ultrasound and MRI examinations revealed the presence of nodules in AFB_1-treated Gnmt-/- mice as early as 9.5 and 11.5 months of age (12.7±2.4 months in males, 12.0±0.6 months in females) while the solvent-treated Gnmt-/- mice as early as 17.6 and 16.5 months of age. The mean age of AFB_1-treated Gnmt-/- mice with nodules was significantly earlier than the solvent-treated Gnmt-/- mice, both in male and female mice ($p<0.05$). Nodules were detected in 5 of 5 female and 6 of 8 male Gnmt-/- mice in the AFB_1 treatment group, but not in any of the female AFB_1-treated wild-type mice and in any solvent-treated wild-type mice. Mice with nodules larger than 0.5 cm were sacrificed for pathological examination and RNA analysis; those without nodules were reexamined periodically. Detailed descriptions of nodule numbers, sizes, and pathologies are shown in Table II.

Among the sacrificed Gnmt-/- mice in the AFB_1 treatment group, 5 of 8 (62.5%) of the males and 4 of 5 (80%) of the females were eventually diagnosed with HCC while 4 of 9 (44.4%) of the males and 8 of 8 (100%) of the females solvent-treated Gnmt-/- mice were eventually diagnosed with HCC. Dysplastic hepatocellular nodules were detected in 3 of 5 (60%) AFB_1-treated male wild-type mice at 20.5 months of age, but not in any female mice treated with AFB_1. In addition to higher

numbers of dysplastic hepatocellular nodules in male compared to female AFB_1-treated Gnmt-/- mice, we also observed higher liver weight/total body weight ratios in males (11.4%) compared to females (7%).

Pathological findings for Gnmt-/- mice.

As shown in Table III and Figure 1*a*, steatosis was present in the liver sections of male AFB_1-treated wild-type mice at the age of 3 months, and necrosis and anisonucleosis were observed at the age of 6 months. In comparison, male AFB_1-treated Gnmt-/- mice developed inflammation (chronic hepatitis), anisonucleosis, and necrosis at 3 months of age and inflammation, focal liver necrosis, and steatosis by the age of 6 months. Interestingly, we observed milder inflammation and necrosis in 6-month-old mice than in 3-month-old of Gnmt-/- mice.

In AFB_1-treated females, inflammation and necrosis were also observed in wild-type mice at the age of 3 months, and microvascular changes were observed by the age of 6 months. Female AFB_1-treated Gnmt-/- mice were diagnosed with inflammation and nuclear dysplasia at the age of 3 months. By 6 months of age they presented inflammation, focal necrosis, bile ductular proliferation, and steatosis (Table III and Fig. 1*a*).

Data from MRI, gross pathology, H&E staining, and reticulin staining

examinations of AFB_1-treated Gnmt-/- mice with HCC are shown in Figure 1*b*. Multiple nodules were found in the livers of both male and female Gnmt-/- mice. Results from the H&E staining of histological mouse liver sections indicate HCC, steatosis, and inflammation in both male and female mice (Table III). Reticulin staining results reveal a loss of reticular fiber in both genders of AFB_1-treated Gnmt-/- mice, with more significant losses noted in males.

Ki-67 staining of Gnmt-/- mice.

Ki-67 staining was used to determine liver cell kinetics in solvent- and AFB_1-treated Gnmt-/- and wild-type mice. In the solvent treatment group, a significantly larger amount of Ki-67-positive cells were found in female Gnmt-/- mice compared to female wild-type mice, but no significant difference was found between male Gnmt-/- and wild-type mice. In the AFB_1 treatment group, significantly larger amounts of Ki-67-positive cells were also found in female Gnmt-/- mice compared to female wild-type mice. In addition, a larger amount of Ki-67-positive cells were found in AFB_1-treated wild-type mice than in solvent-treated wild-type mice. In the Gnmt-/- mice group, there were no significantly difference between AFB_1-treated Gnmt-/- mice and solvent-treated Gnmt-/- mice (Fig. 1*c and d*).

Liver function of Gnmt-/- mice.

Gnmt-/- and wild-type mice sacrificed at 3, 6, and more than 12 months of age were used for liver weight/total body weight data. We found that mean liver weight/total body weight ratios for male and female Gnmt-/- mice treated with tricaprylin (solvent) were significantly higher than those of solvent-treated wild-type mice for all three age groups (Fig. 2), in agreement with previous findings reported by Liu *et al*. [19] With one exception (6-month-old male mice), significantly higher ratios were not found for wild-type mice treated with AFB_1 compared to those treated with solvent. Also, the liver weight/total body weight ratios of AFB_1-treated Gnmt-/- mice were not significantly higher than those of solvent-treated Gnmt-/- mice (Fig. 2). In other words, no correlative effect was observed in either wild-type or Gnmt-/- mice treated with AFB_1 in terms of this ratio.

Mean ALT levels in 3-month-old male and female AFB_1-treated wild-type mice were significantly higher than those in solvent-treated wild-type mice ($p<0.05$) of the same gender, but no statistically significant differences in ALT levels were noted between Gnmt-/- mice treated with solvent and treated with AFB_1 for either gender (Fig. 3). In addition, mean ALT levels in solvent-treated Gnmt-/- mice were significantly higher than in solvent-treated wild-type mice ($p<0.05$).

At 6 months of age, mean ALT levels in female AFB_1-treated wild-type mice were

significantly higher than those in solvent-treated female wild-type mice. Mean ALT levels in AFB_1-treated male and female wild-type mice sacrificed at 12 months of age were significantly higher than those of solvent-treated wild-type mice ($p<0.05$) of the same gender, but no statistically significant differences were found between Gnmt-/- mice treated with either solvent or AFB_1 across genders (Fig. 3).

Microarray analyses of AFB_1-treated wild-type and Gnmt-/- mice.

Microarray analyses using the same protocols described above were performed on mice treated with AFB_1 and sacrificed at 11 weeks of age. AFB_1-treated mouse specimens were labeled as follows: Gnmt-/- A-M, male Gnmt-/-; Gnmt-/- A-F, female Gnmt-/-; WTA-M, male wild-type; and WTA-F, female wild-type. Tests were performed using pooled RNA collected from 3 individuals.

Results from a hierarchical clustering analysis indicate that the Gnmt-/- A-M gene expression pattern clustered with WTA-F and WTA-M, then clustered with WT-F (untreated female wild-type) and WT-M (untreated male wild-type) mice (Fig. 4*a*). Furthermore, Gnmt-/- A-F clustered with Gnmt-/- F (untreated female Gnmt-/-), and then clustered with Gnmt-/- M (untreated male Gnmt-/-). Details for our WTF, WTM, Gnmt-/- F and Gnmt-/- M microarray data are in Liao *et al*.[20] Among the 21,924 analyzed genes, 529 and 192 were up-regulated at least two-fold in female and male

Gnmt-/- mouse livers, respectively, and 74 were up-regulated in both male and female Gnmt-/- mice. In contrast, 417 and 311 genes were down-regulated at least two-fold in female and male Gnmt-/- mouse livers, respectively, and 93 were down-regulated in both male and female Gnmt-/- mice (Fig. 4*b*).

To elucidate Gnmt-/- mice tumorigenesis mechanisms, we used the Babelomics web tool and KEGG pathways database to analyze gene expression patterns among various pathways. Numbers and percentages of significantly deregulated genes with known biological functions are listed separately in Table VI for male and female Gnmt-/- mice. Regardless of gender, higher percentages of deregulated genes were observed in cytochrome P450 and the PPAR signaling pathway. Compared to male Gnmt-/- mice, female Gnmt-/- mice had higher percentages of deregulated genes in the following pathways: lipid metabolism, amino acid metabolism, carbohydrate metabolism, cytokine-cytokine receptor interaction, MAPK signaling, insulin signaling, and focal adhesion. The top three deregulated pathways in AFB_1-treated Gnmt-/- mice were PPAR signaling, cytochrome P450, and the lipid metabolism pathway. We focused on the cytochrome P450 pathway to study AFB_1-induced hepatocarcinogenesis.

Real-time PCR analysis of detoxification pathway genes.

We used real-time PCR analysis to determine the mRNA levels of five genes involved in the cytochrome P450 (Cyp) pathway: Cyp1a2, Cyp3a44, Cyp2d22, Gsta4 (glutathione S-transferase A4) and Abca8a (ATP binding cassette a8a). Compared to solvent-treated wild-type mice, the mRNA levels of these five genes were down-regulated in solvent-treated Gnmt-/- mice. In addition, Cyp1a2, Cyp2d22, Gsta4, and Abca8a mRNA levels in solvent-treated Gnmt-/- mice were similar to those in AFB_1-treated wild-type mice and AFB_1-treated Gnmt-/- mice. According to these data, the effects of AFB_1 treatment on detoxification gene expression profiles were similar to the effects of GNMT deficiency.

Discussion

AFB_1 is the most hepatotoxic and most potent hepatocarcinogenic natural compound ever characterized in humans, non-human primates, and animals such as birds, fish, and rodents.[25] Its ingestion is considered a major risk factor in many countries where HCC is common. We have recently completed work aimed at clarifying GNMT-AFB_1 interaction mechanisms, and suggest that GNMT over-expression in mouse livers provides chemoprevention following treatment with AFB_1.[12] In this study, we used the Gnmt-/- mouse model to demonstrate that Gnmt-/- mice are more susceptible to AFB_1 carcinogenesis than the wild-type mice. It indicates the important role that GNMT plays in liver tumorigenesis caused by AFB_1.

As shown in Table II, the presence of nodules in AFB_1-treated Gnmt-/- mice was observed as early as 9.5 and 11.5 months of age (12.7±2.4 months in males, 12.0±0.6 in females). The age of AFB_1-treated Gnmt-/- mice with nodules was significantly earlier than the solvent-treated Gnmt-/- mice, both in male and female mice. Although the percentage were 100% in female AFB_1 and solvent Gnmt-/- mice, nodules appeared 4-5 months earlier in Gnmt-/- mice treated with AFB_1 than those treated with solvent. It revealed the higher susceptibility to AFB_1-related HCC in Gnmt-/- mice. Possible factors involved in HCC development include abnormal DNA methylation in Gnmt-/- mice[20] and the epigenetic inactivation of gene expression

levels in the detoxification pathway.[26] We determined that the five genes involved in the cytochrome P450 pathway (Cyp1a2, Cyp3a44, Cyp2d22, Gsta4 and Abca8a) were down-regulated in Gnmt-/- mice (Fig. 5). We therefore suggest that GNMT deficiency in mouse livers accelerates AFB_1-induced liver tumor formation by down-regulating gene expression levels tied to detoxification pathways (Fig. 5).

In terms of gender differences in AFB_1-treated Gnmt-/- mice, although the proportion of males having liver tumors was lower than that for females, nodules and pathologies in male mice were much higher in numbers and more severe (Table II). This explains the observed difference in liver tumor formation in Gnmt-/- mice treated with solvent, and indicates that the effect of AFB_1 is stronger in male than in female Gnmt-/- mice. This finding supports Ghebranious and Sell's[9] observation of more significant liver tumor formation in male than in female wild-type mice treated with AFB_1.

The pathological findings also indicate that liver cell lesions in AFB_1-treated Gnmt-/- mice were more serious than in their wild-type counterparts (Fig. 1a). The presence of liver cell lesions in Gnmt-/- mice is likely to trigger regenerative cell proliferation that accelerates chemical carcinogenesis in the liver. In a previous study we reported that Gnmt-/- mice have higher levels of serum ALT, accompanied by histological lesions (coagulation necrosis and liver cell degeneration).[19] In the present

study, Ki-67 staining was used to determine liver cell kinetics in solvent- and AFB_1-treated Gnmt-/- and wild-type mice. Our data indicates larger amounts of Ki-67-positive cells in female Gnmt-/- mice compared to female wild-type mice, but no difference was found between male ones. This data confirms more serious pathological effects for female than for male Gnmt-/- mice.[19] In addition, in the AFB_1-treated group larger amounts of Ki-67-positive cells were noted in female Gnmt-/- mice compared to female wild-type mice, indicating greater sensitivity to AFB_1 exposure among Gnmt-/- mice. Furthermore, larger amounts of Ki-67-positive cells were noted in AFB_1-treated female Gnmt-/- mice than in solvent-treated female Gnmt-/- mice (Fig. 1*c* and *d*). This data demonstrated that the female Gnmt-/- mice were loss the prevention ability when exposure to AFB_1. In short, our results indicate a correlative effect for combined Gnmt deficiency and AFB_1 exposure.

According to the results of our hierarchical clustering analyses, Gnmt-/- A-M (AFB_1-treated male Gnmt-/- mice) gene expression patterns clustered with WTA-F and WTA-M prior to clustering with WT-F and WT-M (Fig. 4). According to these data, the AFB_1 effect was stronger than the GNMT deficiency effect in male mice. The same results show that Gnmt-/- A-F clustered with Gnmt-/- F before clustering with Gnmt-/- M. Combined, these data suggest a significant gender difference in the etiology of liver tumor formation in AFB_1-treated Gnmt-/- mice, with males more

sensitive to AFB_1 and females more sensitive to GNMT deficiency.

As shown in Table VI, higher percentages of deregulated genes in AFB_1-treated Gnmt-/- mice were observed in the cytochrome P450, lipid metabolism, and carbohydrate metabolism pathways, especially in females. In a previous study, we found that Gnmt-/- mice had GSD in their livers, hypoglycemia, and increased serum cholesterol.[19] Other researchers have described a link between HCC and hepatocellular glycogen metabolism, which is associated with the over-expression of several insulin signaling cascade components.[27, 28] As stated above, we observed liver cell lesions in our Gnmt-/- mouse model, especially in females.[19] Such lesions would increase sensitivity to AFB_1 exposure. Our future plans include determining correlations between the carbohydrate metabolism pathway and GSD, GNMT, and AFB_1.

We previously reported a higher mean ratio of liver weight/body weight in Gnmt-/- mice compared to wild-type mice.[19] In the present study, the liver weight/total body weight ratio for AFB_1-treated Gnmt-/- mice did not exceed the ratio for Gnmt-/- mice treated with solvent (Fig. 2)—in other words, no synergy was noted between AFB_1 and Gnmt deficiencies in terms of this ratio. For adult wild-type mice of either gender, the liver weight/total body weight ratio was about 4-5%, indicating that it is worthwhile to compare this ratio at different ages in adult mice.

In terms of liver function, ALT levels in solvent-treated Gnmt-/- mice exceeded those in wild-type mice (Fig. 3). The ALT levels of AFB_1-treated wild-type mice exceeded those found in solvent-treated wild-type mice, but ALT levels in AFB_1-treated Gnmt-/- mice did not exceed those of solvent-treated Gnmt-/- mice at a statistically significant level, indicating that liver damage in AFB_1-treated Gnmt-/- mice was less severe than in solvent-treated Gnmt-/- mice at the ages of 3, 6 and >12 months. Note also that ALT levels in Gnmt-/- mice treated with either AFB_1 or solvent exceeded ALT levels in wild-type mice treated with AFB_1, indicating a more serious effect from GNMT deficiency compared to AFB_1 treatment.

To date, evaluations of AFB_1-related HCC susceptibility have been conducted in a piecemeal fashion, with only a small number of genes considered in terms of their potential participation in the AFB_1-exo-8,9-epoxide detoxifying pathway.[29, 30] Of the Cyp enzymes, Cyp 1A2 and Cyp 3A4 play major roles in AFB_1 bioactivation.[30] According to at least one study, Cyp 1A2 is the primary enzyme for AFB_1-epoxide formation in human livers at a relatively low concentration of AFB_1 exposure.[31] CYP 3A4 metabolizes AFB_1 to AFB_1-exo-8,9-epoxide; the main product of this enzymatic action is aflatoxin Q1 (AFQ_1), a less toxic metabolite.[30] Cyp2d22 expression levels have been described as most abundant in liver tissues,[32] and the Cyp2d22 homologene (CYP2D6 in humans) has been shown to play an important role in AFB_1

bioactivation.[33] In addition, a significant connection has been reported between a Gsta4 polymorphism and AFB_1-induced HCC.[29] Abca8a, which is expressed in high concentrations in the liver, plays an important role in the detoxification pathway.[34] This explains our decision to use these genes to study gene expression profiles in AFB_1- or solvent-treated Gnmt-/- and wild-type mice.

Gene expression profile data gathered for the present study indicate that the mRNA levels of Cyp1a2, Cyp3a44, Cyp2d22, Gsta4 and Abca8a were significantly down-regulated in both male and female Gnmt-/- mice treated with solvent-especially compared to solvent-treated wild-type mice. Furthermore, mRNA levels of Cyp1a2, Cyp2d22, Gsta4 and Abca8a in Gnmt-/- mice treated with solvent were similar to those measured in wild-type mice treated with AFB_1. In contrast, the detoxification ability in Gnmt-/- mice was less than that in wild-type mice, which may explain, at least in part, why liver tumor formation in AFB_1-treated Gnmt-/- mice occurred earlier than in AFB_1-treated wild-type mice. Cyp1a2, Cyp3a44, and Cyp2d22 are primary AFB_1 metabolic enzymes involved in the phase I detoxification pathway.[31, 35] Gsta4 and Abca8a contribute to the phase II detoxification pathway via carcinogen conjugation; they facilitate dissolution in aqueous cellular and extracellular media and elimination from the body. In this study we observed the down-regulation of these five genes in Gnmt-/- mice, indicating that their detoxification capability is sensitive

to AFB_1 exposure. Moreover, the presence of liver cell lesions in Gnmt-/- mice[19] inevitably triggers regenerative cell proliferation, which accelerates chemical carcinogenesis in the liver. Cell proliferation results from Ki-67 staining serve as additional evidence in support of this conclusion (Fig. 1*c*).

In addition, we also observed that the expression levels of Cyp la2 and Cyp2d22 in the AFB_1-treated wild type male mice were lower than the solvent-treated wild type male mice. The down-regulation of Abca8a and Gsta4 in both male and female AFB_1-treated wild type mice was also measured. This data do not agree with a previous report of up-regulated Cyp and GST expression levels following treatment with AFB_1.[36] We believe the difference can be explained in terms of acute and chronic effects. Mice used for real-time PCR analyses were treated with AFB_1 at 7 days and 9 weeks of age and sacrificed at 3 months of age. The pathological data indicate liver cell lesions (steatosis, inflammation, and necrosis) at 3 months. These lesions may have resulted in the down-regulation of Cyp1a2, Ctp2d22, Abca8a, and Gsta4 in AFB_1-treated wild-type mice.

In summary, liver tumor formation in our AFB_1-treated Gnmt-/- mice occurred earlier than in solvent-treated Gnmt-/- mice and AFB_1-treated wild-type mice. In a previous report we described GNMT influence on AFB_1 metabolism and the blocking of its carcinogenic effect.[12] In this study we found that GNMT deficiency increased

susceptibility to AFB_1-related HCC. In addition, expression levels for all five genes involved in the detoxification pathway were down-regulated in Gnmt-/- mice, indicating that GNMT deficiency and AFB_1 exert a correlative effect on carcinogenesis.

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Figure Legends

FIGURE 1 — Representative results from pathological examinations of wild-type and Gnmt-/- mouse livers. (*a*) H&E staining of liver organs from wild-type and Gnmt-/- mice livers at 3 and 6 months of age (upper: 100x magnification; under: 200x magnification). Arrows indicate the pathological finding indicated under the photography. (*b*) MRI, gross pathology, H&E, and reticulin staining of liver organs from AFB_1-treated Gnmt-/- mice 12 months of age and older. (*c*) Ki-67 staining of liver organs from AFB_1-treated wild-type and Gnmt-/- mice. (*d*) The numbers of Ki-67 positive cells of liver organs from solvent- and AFB_1-treated wild-type and Gnmt-/- mice.

FIGURE 2 — Comparisons of liver weight/total body weight ratios for mice with different genotypes at ages 3, 6 and >12 months following different treatments. WTT, wild-type mice treated with solvent; Gnmt-/- T, Gnmt-/- mice treated with solvent; WTA, wild-type mice treated with AFB_1; Gnmt-/-A, Gnmt-/- mice treated with AFB_1. *, $p < 0.05$; **, $p < 0.01$ (Wilcoxon rank sum test).

FIGURE 3 — Comparisons of serum ALT levels for mice with different genotypes at ages 3, 6 and >12 months following different treatments. WTT, wild-type mice treated with solvent; Gnmt-/- T, Gnmt-/- mice treated with solvent; WTA, wild-type

mice treated with AFB_1; Gnmt-/-A, Gnmt-/- mice treated with AFB_1. *, $p < 0.05$ (Wilcoxon rank sum test).

FIGURE 4 — Microarray data evaluations. (*a*) Results from hierarchical cluster analyses of liver tissues from untreated male Gnmt-/- mice (Gnmt-/- M), untreated female Gnmt-/- mice (Gnmt-/- F), untreated male wild-type mice (WT-M), untreated female wild-type mice (WT-F), AFB_1-treated male Gnmt-/- mice (Gnmt-/- A-M), AFB_1-treated female Gnmt-/- mice (Gnmt-/- A-F), AFB_1-treated male wild-type mice (WTA-M), and AFB_1-treated female wild-type mice (WTA-F). Individual probe sets with F p-values <0.001 were clustered using dChip and scaled by color (red, high expression; blue, low expression). Red and blue scales indicate increased or decreased expression levels relative to mean $\log^2$ expression in all 10 tissue samples. (*b*) Venn diagram showing significant changes in the genes of 11-week-old Gnmt-/- mice relative to WT mice. In all, 218 and 324 readable genes were down-regulated in males and females, respectively, and 118 and 455 were up-regulated, also respectively.

FIGURE 5 — Results from real-time polymerase chain reaction analyses of mRNA expression levels in genes involved in the detoxification pathway. *, $p < 0.05$

(Student's *t* test).