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Susceptibility to Aflatoxin B_1-related Primary Hepatocellular Carcinoma in Mice and Humans

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ABSTRACT

The genetic basis of disease susceptibility can be studied by several means, including research on animal models and epidemiological investigations in humans. The two methods are infrequently used simultaneously, but their joint use may overcome the disadvantages of either method alone. We used both approaches in an attempt to understand the genetic basis of aflatoxin B_1 (AFB_1)-related susceptibility to hepatocellular carcinoma (HCC). Ingestion of AFB_1 is a major risk factor for HCC in many areas of the world where HCC is common. Whether humans vary in their ability to detoxify the active intermediate metabolite of AFB_1, AFB_1-*exo*-8,9-epoxide, is not certain but may explain why all exposed individuals do not develop HCC. To determine whether human variability in detoxification may exist, in a study of 231 HCC cases and 256 controls, we genotyped eleven loci in two families of AFB_1 detoxification genes; the glutathione *S*-transferases (GSTs) and the epoxide hydrolases (EPHX). After adjustment for multiple comparisons, only one polymorphism in the epoxide hydrolase family 2 locus remained significantly associated with HCC (odds ratio = 2.06, 95% confidence interval = 1.13–3.12). To determine whether additional susceptibility loci exist, we developed a mouse model system to examine AFB_1-induced HCC. Susceptibility of 7-day-old mice from two common inbred strains (C57BL/6J, DBA/2J) was assessed. DBA/2J animals were 3-fold more sensitive to AFB_1-induced HCC and significantly more sensitive to AFB_1 acute toxicity than were C57BL/6J animals. Analysis of the xenobiotic metabolizing genes in the two strains revealed single nucleotide polymorphisms in three genes, *Gsta4*, *Gstt1*, and *Ephx1*. Although the GSTT1 and EPHX1 loci did not appear to be related to HCC in the total population of the human study, a polymorphism in GSTA4 was significantly related to risk in the male subset. The mouse model also demonstrated that absent or compromised p53 was not necessary for the development of carcinogenesis. These results indicate that the comparison of results from human studies and the AFB_1-susceptible mouse model may provide new insights into hepatocarcinogenesis.

INTRODUCTION

HCC[3] is the fifth most commonly occurring cancer in the world and the third greatest cause of cancer mortality (1). In China, where 54% of HCCs develop, the major risk factors are chronic infection with the HBV and ingestion of foodstuffs contaminated with AFB_1 (2). AFB_1 is a hepatotoxic mycotoxin elaborated by fungi of the *Aspergillus* species that grows readily on foodstuffs stored in damp conditions. Once ingested, AFB_1 is metabolized to an active intermediate, AFB_1-*exo*-8,9-epoxide, which is later detoxified through a variety of metabolic processes. The intermediate epoxide has been shown to bind and damage DNA, primarily at the N7 position of guanine (3). The characteristic genetic change associated with AFB_1, a G→T transversion (4), affects the p53 gene in >50% of the tumors from AFB_1-endemic areas.

Despite the high risk conferred by HBV and AFB_1, not all individuals with these factors appear to have the same risk of developing HCC. It is estimated that only 1 in 20 HBV carriers (people who are seropositive for HBsAg for at least 6 months) will develop a tumor. Although the comparable figure for AFB_1 is more difficult to determine, it is clear that not all individuals who consume AFB_1-contaminated foodstuff have the same risk of HCC. Although studies have reported a significant association between AFB_1 markers in biosamples and HCC, the same studies have failed to demonstrate a correlation between AFB_1 in foodstuffs and AFB_1 biomarkers (5). These results suggest that differences in an individual's abilities to metabolize AFB_1 may be related to HCC susceptibility.

A genetic basis for susceptibility to HCC through variation in genes associated with AFB_1 metabolism has been studied in a number of ways. We and others (6–16) have used genetic mapping methods to examine whether genetic polymorphisms in loci in the AFB_1 detoxification pathways were associated with both AFB_1-adduct levels and HCC in humans. To date, however, these efforts have explored only a small number of genes potentially involved in the detoxification of AFB_1. Moreover, although this approach has many advantages, it lacks precise information concerning AFB_1 exposure and is not amenable to direct experimental manipulation. In addition, although genetic mapping can determine the genetic location and suggest which genes and their variants are correlated with disease, it is incapable of determining causality.

An alternative method for determining genetic susceptibility to AFB_1-related HCC, animal studies, offers several advantages. Animals are experimentally manipulable, the research can be completed in a short period of time, and the studies are capable of determining which components are critical for carcinogenicity. To take advantage of both methods, we conducted parallel HCC studies in humans and in mice. In humans, we extended our previous mapping efforts by examining a comprehensive collection of genetic polymorphisms that are putatively involved in the AFB_1 detoxification pathways. In the mouse studies, we examined the influence of AFB_1 on tumor development in inbred animals to determine whether genes in AFB_1 detoxification differ between susceptible and nonsusceptible strains. We also examined the separate and combined effects of AFB_1, HBV, and mutant p53 on HCC development. The overall goals of the mouse studies were to confirm the human findings and provide a system where the associations could be directly tested.

MATERIALS AND METHODS

Mice. DBA/2J mice were selected as a putative susceptible strain because of their extensive use in hepatocarcinogen sensitivity assays and mapping of HCC susceptibility genes. p53 knockout mice (B6.129S2-Trp53^{tm1Tyj}) were

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; AFB_1, aflatoxin B_1; HBsAg, hepatitis B surface antigen; GST, glutathione *S*-transferase; EPHX, epoxide hydrolase family; GSTP1, GST π1; GSTT1, GST θ1; GSTT2, GST θ2; MGST1, microsomal GST1; GSTA1, GST α1; GSTA4, GST α4; GSTM1, GST μ1; GSTM2, GST μ2; GSTM3, GST μ3; microsomal epoxide hydrolase; OR, odds ratio; CI, confidence interval; SNP, single nucleotide polymorphism; UTR, untranslated region; mEH, microsomal epoxide hydrolase.

included in the experiment to determine whether preexisting mutations in the p53 tumor suppressor gene would increase HCC incidence. Compound heterozygous mice were generated by breeding the p53 KO mice with the HBV HBsAg-transgenic mice (C57BL/6J-TgN(Alb1HBV)^{44Br}) to determine whether the combination of both factors would have a synergistic effect on HCC incidence. C57BL/6J mice were used as a putative hepatotoxin-resistant strain and as a control for the p53 and HBsAg experiments. C57BL/6J, DBA/2J, B6.129S2-Trp53^{tm1Tyj} (K17), and C57BL/6J-TgN(Alb1HBV)^{44Bri} (K18) mice were obtained from Induced Mutant Resource and the breeding colonies of the Jackson Laboratory (Bar Harbor, ME). The C57B6/NCr mice used in the measurement of epoxide hydrolase activity were obtained courtesy of Jeff Green (Center for Cancer Research/National Cancer Institute/NIH).

Chemicals. AFB_1 and tricaprylin were obtained from Sigma (St. Louis, MO). AFB_1 was dissolved in tricaprylin to a final concentration of 400 μg/ml by heating at 65°C and was stored at 4°C until use.

Aflatoxin Analysis. An aflatoxin sensitivity assay was performed by exposing 7-day-old animals to 6 μg/g body weight of AFB_1 in a bolus injection. Female animals were euthanized at weaning. Male animals were then aged for 12 months, sacrificed, and their livers were harvested for histopathology analysis. The livers were examined macroscopically for tumors, then fixed in neutral-buffered formalin, paraffin-embedded, and nonadjacent serial sections were examined for the presence of HCC.

Sequencing. Liver RNA was isolated using Trizol LS (Life Technologies, Inc., Gaithersburg, MD) following the manufacturers recommended protocol. Reverse transcription reactions were performed with the Thermoscript RT-PCR kit (Life Technologies, Inc.). PCRs were performed in an MJ Tetrad Thermocycler (Watertown, MA) with AmpliTaq Gold (ABI Biosystems, Foster City, CA) following the recommended protocol. Sequencing was performed on an ABI 310-automated fluorescent sequencer using the BigDye Terminator sequencing kit (ABI Biosystems). Primer sequences are provided in Appendix 1.

Measuring Epoxide Hydrolase Activity. Three DBA/2J and C57B6/NCr adult male mice were euthanized with CO_2; their livers were removed and snap frozen. A piece of liver the size of a pea was homogenized in 500 μl of protein lysis buffer [1× PBS with 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.004% NaF, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 mM sodium orthovanadate (pH 7.4)]. The homogenates were transferred to a fresh Eppendorf tube, spun for 15 min at 14,000 rpm, and supernatants were transferred to a fresh Eppendorf tube, chilled on ice, centrifuged at 14,000 rpm for 15 min; supernatants were transferred to a fresh Eppendorf, chilled on wet ice, spun at 14,000 rpm for 20 min; supernatants were transferred to a fresh Eppendorf tube and stored at −80°C until use. An aliquot of each sample was used with the BCA Kit (Pierce) to determine concentration. Samples were diluted to 5 μg/μl in ddH_2O.

The epoxide hydrolase activity assay is a slight variation of the Omiecinski assay (17), which is based on a method known to be specific for the microsomal form of epoxide hydrolase. To siliconized plastic, microcentrifuge tubes were added: solution A (0.15 M KCl, 0.05 M Kh_2PO_4), supplemented with 10.0 μl of 3.7 mM (+)-benzo(*a*)pyrene-4,5-epoxide in acetone, 5.0 μl of 0.3 M 1-chloro-2,4-dinitrobenzene in acetone, 0.75 μl of 0.02 M EDTA, and 5 or 10 μg of protein for a total volume of 150 μl. Tubes were incubated at 37°C for 30, 60, or 90 min, after which tubes were immediately transferred to ice. A total of 750 μl of actone:hexane (1:4 v:v) was added, the tubes were vortexed for 60 s, chilled on ice for 5 min, centrifuged at 14,000 rpm for 1 min, and the organic layers were transferred to fresh siliconized microcentrifuge tubes. Samples were reextracted as above using 750 μl of ethyl acetate, organic layers from each sample were pooled, chilled, then dehydrated to dryness and stored at −80°C. High-performance liquid chromatography analysis was conducted as described by Omiecinski *et al.* (17) with a single modification: for the quantitation of epoxide, samples were diluted 40-fold and reanalyzed.

Human Study. The human research was carried out as a nested case-control study within a prospective study of HCC in Haimen City, China, a high-rate area for HCC. Between February 1992 and December 1993, 83,885 adults were enrolled in the cohort (18). Among all cohort members, 48,934 donated a blood sample spotted onto a neonatal screening card.

Two hundred thirty-one individuals who developed HCC during follow-up were matched on age, sex, and township of residence to 256 control individuals. Male cases (n = 187) were matched at a 1:1 ratio. Female cases (n = 44) were matched at a 1:1.5 ratio to increase power. The mean age of the male cases was 55.8 years, and the mean age of the female cases was 59.3 years. There was no difference in occupation between the cases and controls; 88% of the cases and 82% of the controls classified themselves as farmers. Although AFB_1 levels were not measured in the study, Haimen City and environs have been shown to have high AFB_1 levels in prior investigations (19, 20).

HCC diagnosis was confirmed by elevation of serum α-fetoprotein level (>400 ng/ml) in 1.5%, imaging (ultrasonography, computerized tomography, or magnetic resonance imaging) in 39.5%, α-fetoprotein elevation and imaging in 35.8%, clinical criteria in 1.7%, and by death certificate and/or postmortem interview with doctors or family in 21.5%. In addition to these criteria, 8.8% of the diagnoses were also confirmed by histological examination.

HBV status (HBsAg) of all participants was determined in prospectively collected serum samples using radioimmunoassays. The observed HBsAg carrier rate was 13.7% among the whole cohort and 73% among the HCC cases.

Variation in a total of 11 genes potentially involved with AFB_1 detoxification was examined (Appendix 2). These genes belong to either the GST or EPHX. The genes were GSTP1, GSTT1, GSTT2, MGST1, GSTA1, GSTA4, GSTM1, GSTM2, GSTM3, mEH (EPHX1), and soluble epoxide hydrolase (EPHX2). In all loci except EPHX1, one polymorphism was examined. In EPHX1, two polymorphisms in exon 3 and one polymorphism in exon 4 (His139Arg) were examined. In EPHXI exon 3, in addition to the Tyr^{113}His polymorphism at bp 17673, a polymorphism in close proximity at bp 17693, was also examined because of prior evidence that the Tyr^{113}His polymorphism was not in Hardy-Weinberg equilibrium (21). The two exon 3 polymorphisms were genotyped using a single set of primers. Detailed information on the polymorphisms can be found in Appendix 2.

For all loci, variants typed were either previously described in the literature or obtained through data-mining publicly available sequence data using the SNPpipeline of the National Cancer Institute's Cancer Genome Anatomy Project Genetic Annotation Initiative (22). These variants either represent alterations of speculated functional significance (*e.g.*, GSTM1-null, GSTT2-null, EPHX1 alleles) or are within physical distances of close enough proximity to speculate they would be in linkage disequilibrium with variants, which may be of functional significance.

Forward and reverse PCR primers flanking the loci of interest were designed using the Primer program (obtained from the Whitehead Genome Center). Validation of polymorphisms was done by restriction fragment length polymorphism assays and/or direct sequencing of 10 parents from 5 Centre d'Etude du Polymorphisme Humain families (1331, 1332, 1347, 1362, and 1413) representing 20 independent alleles.

PCR reactions were performed in 5 μl of final volume containing 20 ng DNA, 5 μM forward and reverse primers, 0.1 mM deoxynucleotide triphosphates, and 0.005 units AmpliTaq Gold (Perkin-Elmer) in 10× reaction (Perkin-Elmer) buffer. After denaturation for 10 min at 95°C, the reactions were cycled in a Peltier Thermal Cycler (MJ Research PTC-225) 35 times at 94°C for 30 s (denaturation), 64°C for 30 s (annealing temperatures listed in Table 1), and 72°C for 30 s (extension). This was followed by final extension

Table 1 *Detoxification polymorphisms examined in human study, including 231 cases and 256 controls*

Locus	No. of genotypes	χ^2	P
EPHX1-exon3(1)[a]	3	5.61	0.06
EPHX1-exon3(2)[b]	3	1.99	0.37
EPHX1-exon3(3)[c]	6	11.3	0.046
EPHX1-exon4	3	1.24	0.53
EPHX2	3	10.2	0.02
GSTM1	4	1.16	0.76
GSTM2	2	2.3	0.12
GSTM3[d]	1		
GSTP1	59	68.0	0.17
GST12	3	0.68	0.71
GSTA1	3	2.8	0.09
GSTA4	3	1.14	0.56
GSTT1	4	0.66	0.88
GSTT2	3	1.5	0.46

[a] EPHX1-exon3(1) is the Tyr^{113}His polymorphism alone.
[b] EPHX1-exon3(2) is the A/G polymorphism at bp 17,693 alone.
[c] EPHX1-exon3(3) is both exon 3 polymorphisms together.
[d] GSTM3 was monomorphic.

Table 2 *Statistical significance of human susceptibility loci after collapsing genotypes*

Locus (mouse)	Locus (human)	All (231 cases, 256 controls)				Males (187 cases, 189 controls)			
		χ^2	*P*	OR	95% CI	χ^2	*P*	OR	95% CI
Ephx1	EPHX1-exon3(1)[a]	2.30	0.13	1.57	0.87–2.83	0.42	0.51	1.24	0.6–2.41
Ephx1	EPHX1-exon4	0.21	0.64	1.13	0.68–1.86	0.03	0.85	1.05	0.59–1.85
Ephx2	EPHX2	9.1	0.002	2.06	1.28–3.32	10.6	0.001	2.49	1.42–4.36
Gsta4	GSTA4	0.95	0.33	0.84	0.58–1.20	4.52	0.03	1.55	1.03–2.35
Gstm1	GSTM1	0.95	0.33	0.83	0.57–1.21	0.83	0.36	0.82	0.54–1.25
Gstt1	GSTT1	0.38	0.53	0.88	0.59–1.31	0.02	0.88	0.96	0.62–1.50

[a] Contrasting individuals with CC genotypes *versus* individuals with CT and TT genotypes.

for 10 min at 72°C. Reactions were held at 15°C. Restriction digests were performed using 5 μl of the PCR products, added to 3.5 μl of H_2O, 1.0 μl of 10× buffer, and 0.5 μl of restriction enzyme, and incubated 2 h at 37°C. For most reactions, 2 μl of the total digest was added to 3 μl of loading buffer and run in 2% NuSieve agarose (FMC) in 1× TAE buffer for about 30 min, at 125 V. Gel bands were visualized by GelStar (FMC) staining and UV transillumination. Images were captured with a Kodak DC120 Zoom Digital Camera and the Electrophoresis Documentation and Analysis System 120 (Kodak Digital Science).

In addition to the GST and EPHX polymorphisms, nine microsatellite markers (D13S317, D18S51, D21S11, D3S1358, D5S818, D7S820, D8S1179, FGA, and vWA) were typed to determine the genetic profiles of the study participants (Appendix 3). These nine markers have been extensively used for identification and evolutionary studies in population genetics. In this study, the information on these markers was used as the genetic background of each subject and was used to compare the origins of subjects and adjust for the possible affects of population stratification between cases and controls. Genotyping was performed using Applied Biosystems AmpFESTER Profiler Plus PCR Amplification Kit (PN 4303326). The amplification reaction was carried out in a 5-μl volume containing 10 ng of DNA, 2.0 μl of PCR reaction mix, 0.9 μl of Profiler Plus Primer Set, and 0.1 μl of AmpliTaq Gold polymerase. After 11 min at 95°C to activate the polymerase, 28 cycles of 1 min at 94°C, 1 min at 59°C, 1 min at 72°C was followed, then 45 s at 60°C for extension and hold at 25°C. The products were then run on an ABI Prism 377 with XL Upgrade on 0.2-mm thick 6% polyacrylamide gels.

Statistical Analyses. Contingency table analysis was used to assess associations between genotype and case/control status. To evaluate potential population stratification, logistic regression models were used to evaluate the main effect of nine DNA fingerprinting markers between cases and controls and also to examine the independent effect of polymorphisms on cancer susceptibility genes after adjusting for the genetic background measured by the nine DNA profile markers. The Hosmer-Lemeshow method was used to assess the fit of each logistic model (23). To address the significance of the susceptibility polymorphisms, the −2-log likelihood difference was calculated between the model, including the DNA fingerprint markers and the same model with the addition of one susceptibility polymorphism. The difference follows a χ^2 distribution with one degree of freedom. All analyses were performed using SAS, version 8.0 (SAS Institute, Cary, NC).

RESULTS

Human Results. The results of the polymorphism analyses are presented in Table 1. GSTM3 was observed to be monomorphic in the study population. Among the remaining loci, EPHX2 was significantly associated with HCC ($\chi^2 = 10.2$, $P = 0.02$). This result continued to be significant after a conservative adjustment for multiple comparisons based on testing independent genes. The combined exon 3 polymorphisms in EPHX1 were also significantly associated with HCC ($\chi^2 = 11.3$, $P = 0.046$), but the association lacked biological plausibility because it was based on an underrepresentation of heterozygotes among the cases.

In Table 2, the results of the analysis, collapsed over genotypes and shown separately for males, are presented. The GSTM1 and GSTT1 genotypes were collapsed into null genotype and not null genotype groups. For the EPHX1 polymorphisms, the genotypes were grouped by suggested functionality (21). The EPHX1 exon 3 $Tyr^{113}His$ polymorphism is a T to C replacement that changes tyrosine residue 113 to histidine and has been associated with decreased enzyme activity. In contrast, the EPHX1 exon 4 polymorphism is an A to G replacement that changes histidine residue 139 to arginine and has been associated with increased enzyme activity (21). Thus, EPHX1-exon 3 was collapsed into CC *versus* other, and EPHX1-exon 4 was collapsed into AA *versus* other. For the loci in which there were no *a priori* hypotheses, the individuals who were homozygous for allele 1 were contrasted with the individuals of other genotypes. In the total study population, the EPHX2 polymorphism remained the only polymorphism significantly associated with HCC with an OR of 2.06 (95% CI = 1.28–3.32). When examined separately by gender, the EPHX2 polymorphism was significantly related to HCC risk in men (OR = 2.49, 95% CI = 1.42–4.36). The GSTA4 polymorphism was also related to risk in men (OR = 1.55, 95% CI = 1.03–2.35). Examination of nine microsatellite markers found no evidence of significant population stratification (data not shown). In addition, the results were not altered by adjusting for stratification, using these markers.

Mouse Experiments. A significant difference in acute sensitivity to AFB_1 was observed between mouse strains. The animals with the C57BL/6J genetic background or congenic on C57BL/6J (B6.129S2-$Trp53^{tm1Tyj}$) were resistant to AFB_1-induced mortality (Table 3). Thirty of 32 of the animals of both sexes (94%) survived to weaning. In contrast, DBA/2J mice were highly sensitive to AFB_1-induced mortality, with only 47% of injected animals of both sexes surviving

Table 3 *Effect of AFB_1 exposure on various strains of mice*

Strain	Phenotype	Exposure	No. of pups injected	No. of pups weaned	Percentage surviving to weaning	Animals with HCC	Percentage having HCC
C57BL6/J	HCC resistant	AFB_1	16	15	94%	4	27%
C57BL6/J	HCC resistant	Tricaprylin	7	7	100%	0	0%
DBA/2J	HCC susceptible	AFB_1	21	10	47%	9	90%
DBA/2J	HCC susceptible	Tricaprylin	6	6	100%	0	0%
B6.129S2-$Trp53^{tm1Tyj}$	p53 (−/−)	AFB_1	6	5	83%	0[a]	0%[a]
B6.129S2-$Trp53^{tm1Tyj}$	p53 (+/−)	AFB_1	10	10	100%	1	10%
C57BL/6J-TgN(Alb1HBV)^{44Bri}	HBV(+)	AFB_1	7	7	100%	7	100%
C57BL/6J-TgN(Alb1HBV)^{44Bri} B6/129S2-$Trp53^{tm1Tyj}$	HBV(+)/p53 (+/−)	AFB_1	3	3	100%	3	100%

[a] All animals died of lymphoma.

to weaning. All of the animals injected with tricaprylin, regardless of strain, survived to weaning (7 C57BL/6J and 6 DBA/2J,).

The same strains that were resistant to the acute effects of AFB_1 were also more resistant to developing HCC. Only 4 of 15 C57BL/6J male mice developed HCC. In contrast, by 52 weeks of age, 9 of 10 DBA/2J male mice had developed HCC. Histological examination of the livers of the tumor-free animals of both strains demonstrated the presence of regenerative hyperplasia and atypical nodules indicating that AFB_1 was toxic in both genetic backgrounds but that the DBA/2J mice were more likely to progress to a tumorigenic state.

The potential involvement of p53 in AFB_1-induced HCC was assessed in heterozygous and nullizygous animals. Five nullizygous mice were included in the experiment, however, all succumbed to lymphoma before the 52-week time point. Necropsies did not reveal evidence of HCC in these animals. Of the 10 $p53^{(+/-)}$ mice who survived to weaning, only 1 developed HCC by 52 weeks of age.

As anticipated from previously published studies, AFB_1 exposure accelerated the development of HCC in HBV-transgenic mice. All 7 of the HBV-transgenic mice developed tumors by the 52-week time point. In addition, all compound heterozygous animals [$p53^{(+/-)}$/$HBsAg^+$] developed multifocal tumors. There was no difference in tumor multiplicity or size between the HBV^+ and [$p53^{(+/-)}$/HBV^+] animals suggesting that there was no synergistic interaction between the transgene and the p53 mutation.

To determine whether the AFB_1 sensitivity differences between C57BL/6J and DBA/2J strains could be explained by polymorphisms in the AFB_1-metabolizing pathways, a polymorphism screen was performed. PCR primers were designed to cover the open-reading frame of the known GST and epoxide hydrolase genes. PCR products were generated from genomic DNA or reverse-transcribed liver RNA and sequenced. SNPs between C57BL/6J and DBA/2J were observed in three genes, *Gsta4*, *Gstt1*, and *Ephx1*. The *Gsta4* polymorphism was an A-to-C transversion in the 5′UTR, 37 nucleotides upstream of the ATG translation start site (35A>C). Five different transition SNPs were observed in *Gstt1*; G-to-A in the 5′UTR, 11 bases upstream of the ATG site (88G>A); 257T>C, 432A>G, 435T>C, and 691G>A. None of the *Gstt1*-coding SNPs resulted in an amino acid substitution. The single *Ephx1* polymorphism, 1056C>T, encodes an arginine to cysteine (R338C) substitution. This substitution is observed to alter a highly conserved amino acid (Fig. 1).

The results of the Omiecinski assay (17) to assess epoxide hydrolase activity additionally reinforce the potential functional significant of the *Ephx1* substitution. Liver extracts from the DBA/2J mice consistently showed lower capacity to clear the benzo(*a*)pyrene-4,5-epoxide than did the C57BL/6J containing the conserved amino acid. At the 90-min time point, both the 5 μg of protein sample (0.014% DBA/2J *versus* 0.037% C57BL/6J, $t_{df\ 4} = 4.7$, $P = 0.079$) and 10 μg of protein sample (0.011% DBA/2J *versus* 0.055% C57BL/6J, $t_{df\ 4} = 2.3$, $P = 0.009$) showed lower conversion percentages.

DISCUSSION

To date, the evaluation of AFB_1-related HCC susceptibility has been conducted in a piecemeal fashion. Only a small number of the genes potentially acting in the AFB_1-*exo*-8,9-epoxide detoxifying pathway have been evaluated in humans. Although human studies stimulated by murine results have been previously reported in lung cancer (24), there have been no attempts to validate liver cancer associations observed in humans in model organisms or to use model organisms to identify potential risk loci in humans.

In this study, we combine the power of a systematic, pathway-driven, gene-mapping approach to human association studies with the novel insights gained by jointly evaluating a comparable animal model. HCC is a quintessential complex trait that develops because of a combination of genetic and environmental factors. It is likely that individual genetic effects are small, multiple, and minimally additive. A given set of environmental factors may be neither necessary nor sufficient to cause disease. Human studies have many advantages when exploring complex traits. Foremost among these is that they have direct application of observed associations. They suffer, however, from imprecise measurement of exposure variables, difficulty in performing replication studies, and the reality that correlation does not demonstrate causality. Animal models, although more definitive in causality and useful in demonstrating etiologic mechanisms, may not translate well to humans. A combination of the two approaches, however, reinforces the findings of both.

Concern about the risk of HCC associated with aflatoxin has prompted a great deal of research in humans. Evidence from several sources suggests that the GSTs play a key role in the Phase II metabolism of AFB_1. Although the GSTs have somewhat overlapping specificities, *in vitro* studies of human hepatocytes have suggested that GSTM1 is critical in AFB_1 conjugation in humans. The previous studies that have examined the relationship between GSTM1 genotype and AFB_1-albumin adducts have reported somewhat inconsistent results. Our original article reported a significant relationship between the GSTM1 null genotype and the presence of AFB_1-albumin adducts in Ghanaian men ($P = 0.03$; Ref. 6). Wild *et al.* (25), studying a Gambian population, also reported an association between the GSTM1 null genotype and AFB_1-albumin adducts, although the association was restricted to people who were not infected with HBV ($P = 0.015$). In contrast, an earlier study by the same group found no association between GSTM1 and AFB_1-ablumin levels among Gambian children, although the low frequency (17.7%) of the GSTM1 null genotype may have limited study's power (26). Chen *et al.* (15) reported a significant association between GSTM1 genotype and AFB_1-albumin adducts among residents of Matzu Island, China, although the association was in the direction opposite those of previous studies. Ahsan *et al.* (27) reported no association between GSTM1 genotype and AFB_1-albumin adducts in a Taiwanese population.

Studies of the GSTM1 genotype association with HCC have proven as inconsistent as the studies of GSTM1 genotype and AFB_1-albumin adducts. An association with GSTM1 has been supported by Chen *et al.* (8) and Yu *et al.* (10), reporting on a Taiwanese cohort study, and Bian *et al.* (28), reporting on a Chinese population. Similarly, Omer *et al.* (12) found a significant association between GSTM1 genotype and HCC in Sudan but only in relationship to peanut butter consumption. In contrast, Yu *et al.* (7), Hsieh *et al.* (9), Sun *et al.* (11), and Chen *et*

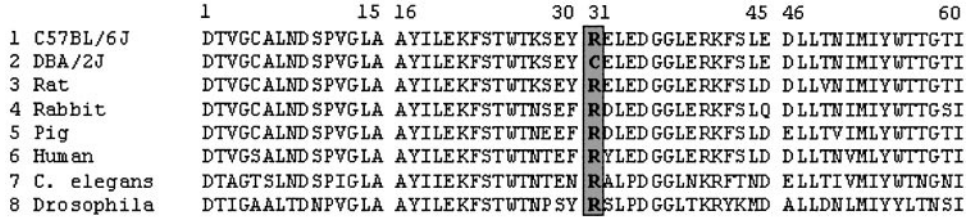

Fig. 1. Evolutionary conservation of AA338 in EPHX1.

al. (14), reporting on Taiwanese populations, found little association between GSTM1 genotype and HCC. Although our original study reported a relationship between the GSTM1 null genotype and HCC, the relationship was of borderline significance ($\chi^2 = 3.4$, $P = 0.06$; Ref. 5). That association, however, was not replicated in the current dataset. Taken as a whole, the data suggest that any association between GSTM1 and HCC is likely to be weak.

The relationship between GSTT1 and HCC has also been examined by several studies in addition to the one currently presented. Chen *et al.* (8), Yu *et al.* (10), and Sun *et al.* (11) all reported significantly increased risk for HCC with GSTT1 null genotype and other risk factors acting in concert. In contrast, GSTT1 genotype had no effect on HCC risk in a Sudanese population (13). In agreement with the latter finding, we found no relationship between the GSTT1 null genotype and risk of HCC in the current dataset. GSTP1 has been examined in one study in addition to the present one. Chen *et al.* (14) reported an association between GSTP1 and PAH-associated HCC, which we did not replicate in the current study of AFB_1-related HCC.

mEH, encoded by EPHX1, catalyzes the hydrolysis of reactive epoxide intermediates, thereby favoring their elimination. Two polymorphisms in EPHX1 have been associated with variation in mEH activity. The substitution of histidine for tyrosine at residue 113 in exon 3 results in decreased mEH activity, whereas the substitution of arginine for histidine at residue 139 in exon 4 results in enhanced mEH activity. Using yeast strains that express human mEH, Kelly *et al.* (29) recently reported that mEH has a functional role in the detoxification of AFB, as measured by DNA adduct formation, mitotic recombination, and Ames assay mutagenicity. Epidemiological associations of EPHX1 polymorphisms with HCC and AFB_1 have been investigated in several studies. In our original study, we found a significant association ($P = 0.01$) between the ^{113}His allele in the exon 3 polymorphism and HCC in a case-control study conducted in Shanghai, China (6). In a companion study reported in the same publication, we also found an association ($P = 0.02$) between the ^{113}His allele and presence of AFB_1-albumin adducts in a Ghanaian population. In contrast, Wong *et al.* (16) reported no association between either the EPHX1 exon 3 or exon 4 polymorphism and HCC among Scottish individuals, a population in which AFB_1 was unlikely to be a risk factor. Tiemersma *et al.* (13) reported associations between both the ^{113}His allele of the exon 3 polymorphism and the ^{139}His allele of the exon 4 polymorphism and HCC in a Sudanese population. However, neither association attained statistical significance and neither affected the relationship between HCC and peanut butter consumption, the major AFB_1-contaminated foodstuff in the population. Consistent with the finding of no association between AFB_1 levels and EPHX1 polymorphisms were data reported by Wild *et al.* (25) studying a Gambian population. In agreement with the latter finding, in our current dataset we find no convincing association between EPHX1 and HCC.

Soluble epoxide hydrolase, encoded by EPHX2, has been studied for its ability to convert epoxyeicosatrienoic acids to dihydroxyeicosatrienoic acid (30). Although polymorphisms in EPHX2 have been examined for a possible relationship to Parkinson's disease (31), variation in EPHX2 has not been previously examined for a role in AFB_1 metabolism or for an association with HCC. Our finding of a significant association among the whole population and among males, alone, suggests that EPHX2 genotype may confer genetic susceptibility to HCC to some segments of the population.

The mouse has been used extensively for the study of genetic factors that influence the carcinogenicity of a variety of hepatotoxins. DBA/2J, C3H/HeJ, C57BL/6J, and F1 hybrids have been used to map a number of loci that modulate the susceptibility to hepatotoxins, including urethane, *N,N*-diethylnitrosamine, and *N*-ethyl-*N*-nitrosourea (32–35). Genetic mapping studies exploring HCC susceptibility to these agents have suggested seven different hepatocarcinoma susceptibility loci, *Hcs1–7* (33, 36–37). Little work, however, has been performed to explore genetic susceptibility to AFB_1-associated HCC. This lack of investigation is likely attributable to the early observation of resistance of adult mice to AFB_1-induced HCC (38, 39). The model used in this study (40) has not been extensively explored and may, in fact, serve as a better analogue for humans in that that human AFB_1 exposure via contaminated foodstuffs begins early in life (41).

In the present study, we have demonstrated that mouse strains susceptible to AFB_1-related HCC vary from nonsusceptible strains at several AFB_1 detoxication loci: Gsta4, Gstt1, and Ephx1. The identification of a member of the Gsta class was consistent with previous speculation that the resistance of adult mice to AFB_1 toxicity may be attributable to the constitutive expression of a Gsta member: Gsta3 (42). Interestingly, EPHX1 and GSTT1 are consistent with the locations of two of the genetically mapped murine HCC susceptibility loci: *Hcs6* and *Hcs7*. Examination of the human counterparts to these genes in the whole population did not find similar associations. However, if one examines the comparable group in humans (*i.e.*, males), GSTA4 variation is observed to be associated with HCC risk (Table 2). The effect of stratifying the human population by HBsAg status to even more closely reflect the mouse model did not achieve statistical significance (data not shown), possibly because of insufficient sample size. It is well known that human HCC risk varies by sex (43). One might speculate that these finding will be useful in elucidating the etiologic basis underlying this risk.

It is provocative that the specific loci of the given gene families that modified the risk of HCC in humans appear to differ from those that modify the risk in mice. This may indicate that different genes within these families are responsible for detoxication in different species. Alternatively, differences in observed loci may suggest that variation in either population is attributable to chance and that the increased susceptibility of the DBA/2J mice is unrelated to variation of the detoxication loci. In the mouse, it is possible to more directly test this later possibility. The alternative mouse strains have been tested to evaluate their capacity to clear the epoxides. Those mice with the at risk genotypes were observed to have significantly reduced capacity to metabolize benzo(*a*)pyrene-4,5-epoxide. Additional genetic studies are under way to assess which of the three loci observed in this study segregate with risk and ability to detoxify epoxides. Once completed, it will be possible to create genetically engineered mice to replace the observed genetic alteration as a direct test of its role in susceptibility.

An additional insight that could be gained from these experiments relates to the role of deleted p53 in AFB_1-exposed mice. No difference was observed between HCC rates in $p53^{(+/-)}$ *versus* $p53^{(+/+)}$ mice. These results suggest that loss of p53 is not rate limiting in AFB_1-associated HCC. Additional larger-scale crosses are under way to create composite genetic models and to examine their corresponding risk.

APPENDIX

Appendix 1 *Primer sequences for mouse loci*

Primer Name	Sequence 5′-3′	Primer Name	Sequence 5′-3′
Ephx1-1F	AGTAGGAACCCGAGAGCGAC	Gstm4-1F	GAGTCTTTCCCGACCAGTGA
Ephx1-1R	CAGCCCTTCAATCTTGGTCT	Gstm4-1R	ACACGGATCCTCTCCTCCTC
Ephx1-2F	GGTGGAGATCCTCAACCAAT	Gstm4-2F	GCACAACCTGTGTGGAGAGA
Ephx1-2R	GGCTATGTTGGTGCAGATGA	Gstm4-2R	CATTCGATCGTAATCAAGGACA
Ephx1-3F	AGTTCTACATTCAAGGCGGC	Gstm5-1F	TAAAGTTAGCCGCCCACAGT
Ephx1-3R	TTGGTCCAGGTGGAGAACTT	Gstm5-1R	CTGAGGCTTCAGGTTTTCGT
Ephx1-4F	GCCTGGCTGCCTACATCTT	Gstm5-2F	AACCAGATCATGGACTTCCG
Ephx1-4R	TAGCGTCATCACTGCAGCTC	Gstm5-2R	CAGTGAGCTAAGAGTGTGGGC
Ephx2-1F	TCCAGCTTCGTGTCTGTGTC	Gstm6-1F	CGTGACTCTGGGTTATTGGG
Ephx2-1R	CAGCCAGTTGTTGGTGACAA	Gstm6-1R	ACTCTGGCTTCCGTTTCTCA
Ephx2-2F	AAAAGAAAGGATTCACAACATGC	Gstm6-2F	GAATTCAGATGGGCATGCTT
Ephx2-2R	GAGCCCATCTCCACAAAATG	Gstm6-2R	TCAGTGTTCACGGTGACAGG
Ephx2-3F	AGGGATCCGCCTGCATTT	Gstm6-3F	TTGGTGGGGAAGATCTATCAG
Ephx2-3R	TCATGGGAGACACATCAGGA	Gstm6-3R	CTCACACAAGACTGGGCCTT
Ephx2-4F	GCTGTGGCCAGTTTGAACAC	Gstp1-1F	AGATGGGGTGGGAAAGTGTA
Ephx2-4R	CCAACCCTTTACAGCTCCAC	Gstp1-1R	CCTCCACTACGGGTGTCACT
Gsta1-1F	AGCCCGTGCTTCACTACTTC	Gstp1-2F	TGAGTGACACCCGTAGTGGA
Gsta1-1R	CAAGGCAGTCTTGGCTTCTC	Gstp1-2R	CAGATGCAGCCTGAACAAGA
Gsta1-2F	TTGGGCAATTGGTATTATGTCC	Gstp1-3F	CCTGAGGAAATCAGGGATCA
Gsta1-3R	AATCTTGAAAGCCTTCCTTGC	Gstp1-3R	CGTGAGGGACACACACACTC
Gsta2-1F	ATTGGGAGCTGAGTGGAGAA	Gstp1-4F	CATTACAGGGCAGCAGGAGT
Gsta2-1R	TTGCCCAATCATTTCAGTCA	Gstp1-4R	GAGGTGTGGGTCTCAGAAGC
Gsta2-2F	GGAGAGAGCCCTGATTGACA	Gstp1-5F	AAGGACAAGAGGGAGCCATA
Gsta2-2R	GTATCTGCGGCTCCATCAAT	Gstp1-5R	AAAACGGGGACAAGAAGCTC
Gsta3-1F	CAAGAAAACCCAAGCAACTG	Gstp2-1F	CCCTCTGTCTACGCAGCACT
Gsta3-1R	TATCTCCAGATCCGCCACTC	Gstp2-1R	CAGGGCCTTCACGTAGTCAT
Gsta3-2F	TGGGAAGGACATGAAGGAGA	Gstp2-2F	GAATGATGGGGTGGAGGAC
Gsta3-2R	CTGCCAGGTTGAAGGAACTT	Gstp2-2R	TACTGTTTGCCATTGCCATT
Gsta3-3F	TGTGGACAACTTCCCTCTCC	Gstt1-1F	GACCTCGTGCTTCCAGAGTC
Gsta3-3R	CCAGAGTCTAAGAAGCTTGTTTTGT	Gstt1-1R	GCGGCACCTTAGAGAATGAC
Gsta4-1F	CTTCTTTCTCGAGTGCCTGG	Gstt1-2F	GATGACCCGTACAAGAAGGC
Gsta4-1R	TCTTTTTCCTTGGGGGTTTT	Gstt1-2R	CAGTGAGGGGAAACAGCATT
Gsta4-2F	ACATGTATGCAGATGGCACC	Gstt2-1F	CAGAGGAGGAAATCGTTTGG
Gsta4-2R	TGACACTGCAATTGGAACCT	Gstt2-1R	GTGGTCTGCCACCTGGTACT
Gstm1-1F	CTTCCGCTTTAGGGTCTGCT	Gstt2-2F	GACGGAAGCTTCGTGTTGAC
Gstm1-1R	AAGGCAGATTGGGAAAGTCC	Gstt2-2R	GGGCTGCATCAACTCCTC
Gstm1-2F	ATACACCATGGGTGACGCTC	Gstt2-3F	GCAACAGCTGGAGGACAAGT
Gstm1-2R	GACCTTGTCCCCTGCAAAC	Gstt2-3R	GGAGGGGGTACTGGTAACAT
Gstm1-3F	TCTACTCTGAGTTCCTGGGCA		
Gstm1-3R	AGAGAGAACCAGGAGCCACA		
Gstm2-1F	TGACACTAGGTTACTGGGACATC		
Gstm2-1R	TTTTCTCAAAGTCAGGGCTGT		
Gstm2-2F	GGAGAACCAGGCTATGGACA		
Gstm2-2R	GGGTTCCAAAAGGCCATC		
Gstm2-3F	CTTTGAGGGCCTGAAGAAGA		
Gstm2-3R	ACCAAGGCAGCACACAGAC		
Gstm2-4F	CTTCCCTCAGTGATGGTTGG		
Gstm2-4R	CATCAAAAGGCTTCCTCTGG		
Gstm2-5F	AAGCGCTGAGAAGCAGAGTC		
Gstm2-5R	CCAGAGTCTAAGAAGCTTGTTTTGT		
Gstm2-6F	AGGTTACTGGGACATCCGTG		
Gstm2-6R	TAGGTGACCTTGTTCCCTGC		
Gstm2-7F	AGTTGGCCATGGTTTGCTAC		
Gstm2-7R	AGAAGAAAGCTGCACGTGGT		
Gstm3-1F	ACTGACTCACTCCATCCGCT		
Gstm3-1R	AGGGATGGCCTTCAAGAACT		
Gstm3-2F	AACCAAGTTATGGACACCCG		
Gstm3-2R	CATATGGACAGTCCCCCACT		
Gstm3-3F	CATGAAGAGTAGCCGCTTCC		
Gstm3-3R	GGAGGGCTGGCACTAAGATA		

Appendix 2 *Detailed information on the polymorphisms in 11 HCC susceptibility genes in humans*

Gene location	Accession no.	SNP: position	Size (bp)	Primer 1	Primer 2	Annealing temperature	Scoring
EPHX1-3e 1q42.1 (exon 3)	L29766 AF253417	Exon 3, codon 113, T to C (Tyr to His) T/C: 17673 A/G: 17693	147	GCTGCTTCCACTATGGCTTC	GGCGTTTTGCAAACATACCT	66°C	11 (CC:GG) 12 (CT:GG) 13 (CT:GA) 22 (TT:GG) 23 (TT:GA) 33 (TT:AA)
EPHX1-4e 1q42.1 (exon 4)	L29766 AF253417	Exon 4, codon 139, A to G, (His to Arg) A/G: 24448	381	CAGAGCCTGACCGTGCAG	GGTCACCCCGCCGGAAGG	67°C	11 (AA) 22 (GG) 12 (GA)
EPHX2 8p21-p12	L05779	A/G: 1742	291	CGGTGGTCTCAAAGATGTAGA	TGTCCCCCTACAGGACACTA	60°C	11 (AA) 22 (GG) 12 (AG)
GSTP1 11q13	M37065	STRs (AAAAT): 1856–1942	230-295	CACGCACCTATAATTCCACC	GCTTAGAGGAAAGGAAATTGC	54°C	NA
GSTT1 22q11.23	Z67376	STRs (CA repeats): 110-155	268	CAACTTCATCCACGTTCACC	GAAGAGCCAAGGACAGTTAC	54°C	NA
GSTT2 22q11.23	Z84718	G/T: 66094	527	TAAAACACTGATGACATTTGCC	AGGTGACACTGGCTGATCTC	56°C	11 (TT) 22 (GG) 12 (TG)
MGST1 12p12.3-p12.1	J03746	G/A: 560	325	TTCCATGGCTTACAGGTTG	AGTGAGGTGTTGTGTGAATGTT	66°C	11 (AA) 22 (GG) 12 (AG)
GSTA1 6p12	L13269	C/T: 3962	287	CCAACCTTGAAAAGGAACAC	CTAGACAGGAGGGTGTAAGGC	66°C	11 (CC) 22 (TT) 12 (CT)
GSTA4 6p12	AL121969	T/G: 97161	397	TGTAAAACGACGGCCAGTGGCCA TAAAACAACACATCC	CAGGAAACAGCTATGACCGAGA GCAGAAAGACGCTCAG	62°C	11 (TT) 22 (GG) 12 (TG)
GSTM1 1p13.3	X68676	C/G: 534	132	GCTTCACGTGTTATGAAGGTTC	TTGGGAAGGCGTCCAAGCGC/ TTGGGAAGGCGTCCAAGCAG	56°C	11 (CC) GSTM1B 22 (GG) GSTM1A 12 (GC) GSTM1AB
GSTM2 1p13.3	M63509	G/A: 905	284	GCCCTTTAAAGCAGACACAA	GAGTGAGGAGCCCATACTCA	56°C	11 (AA) 22 (GG) 12 (GA)
GSTM3 1p13.3	AF043105	AGG/—: 4596–4598	273	CCTCAGTACTTGGAAGAGCT	CACATGAAAGCCTTCAGGTT	58°C	11 GSTM3*A 22 GSTM3*B 12 GSTM3*AB

Appendix 3 *Information on nine microsatellite markers used as genetic background in humans*

Locus designation (location)[a]	Size range (bp)	Dye label	% of homozygosity in different population[b] (F_{ST})			
			African American	Caucasian	Hispanic	Asian
D3S1358 (3p)	114–142	5-FAM (blue)	21.4% (−0.0005)	19.2% (−0.0009)	23.1% (0.0014)	27.6% (0.0035)
vWA (12p12-pter)	157–197	5-FAM	20.9% (0.0011)	24.8% (−0.0011)	16.7% (0.0029)	16.8% (0.0027)
FGA (4q28)	219–267	5-FAM	14.5% (0.0004)	11.9% (−0.0004)	14.1% (0.0008)	10.2% (0.0029)
D8S1179 (8)	128–168	JOE (green)	22.0% (−0.0001)	24.4% (0.0000)	17.9% (0.0005)	23.5% (0.0025)
D21S11 (21)	189–243	JOE	14.5% (0.0005)	18.9% (0.0008)	17.5% (0.0013)	18.9% (0.0056)
D18S51 (18q21.3)	273–341	JOE	11.0% (0.0012)	12.9% (0.0001)	10.3% (0.0011)	7.7% (0.0046)
D5S818 (5q21-31)	135–171	NED (yellow)	29.5% (0.0010)	29.7% (−0.0001)	28.6% (0.0010)	23.0% (0.0028)
D13S317 (13q22-31)	206–234	NED	31.6% (0.0029)	25.6% (−0.0008)	17.5% (0.0047)	29.1% (0.0071)
D7S820 (7q)	258–294	NED	24.2% (0.0000)	18.3% (−0.0005)	20.1% (0.0010)	23.0% (0.0039)

[a] Information on the location, size, and dye label came from the user's manual of Applied Biosystems AmpFESTER Profiler Plus PCR Amplification kit.
[b] Information on percentage of homozygosity and F_{ST} in different populations was summarized from Budowle *et al.* (44).

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