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Genetics

Susceptibility to hepatocellular carcinoma is associated with genetic variation in the enzymatic detoxification of aflatoxin B_1

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ABSTRACT **Aflatoxin B_1 (AFB_1) has been postulated to be a hepatocarcinogen in humans, possibly by causing p53 mutations at codon 249. AFB_1 is metabolized via the phase I and II detoxification pathways; hence, genetic variation at those loci may predict susceptibility to the effects of AFB_1. To test this hypothesis, genetic variation in two AFB_1 detoxification genes, epoxide hydrolase (EPHX) and glutathione *S*-transferase M1 (GSTM1), was contrasted with the presence of serum AFB_1-albumin adducts, the presence of hepatocellular carcinoma (HCC), and with p53 codon 249 mutations. Mutant alleles at both loci were significantly overrepresented in individuals with serum AFB_1-albumin adducts in a cross-sectional study. Mutant alleles of EPHX were significantly overrepresented in persons with HCC, also in a case–control study. The relationship of EPHX to HCC varied by hepatitis B surface antigen status and indicated that a synergistic effect may exist. p53 codon 249 mutations were observed only among HCC patients with one or both high-risk genotypes. These results indicate that individuals with mutant genotypes at EPHX and GSTM1 may be at greater risk of developing AFB_1 adducts, p53 mutations, and HCC when exposed to AFB_1. Hepatitis B carriers with the high-risk genotypes may be an even greater risk than carriers with low-risk genotypes. These findings support the existence of genetic susceptibility in humans to the environmental carcinogen AFB_1 and indicate that there is a synergistic increase in risk of HCC with the combination of hepatitis B virus infection and susceptible genotype.**

Primary hepatocellular carcinoma (HCC) is a tumor that occurs at high frequencies in east Asia and subSaharan Africa. In those areas of the world, chronic infection with the hepatitis B virus (HBV) is the best described risk factor; however, only 20–25% of HBV carriers develop HCC. Exposure to the mycotoxin aflatoxin B_1 (AFB_1) has also been suggested to increase HCC risk (1), in part because *in vitro* experiments have demonstrated that AFB_1 mutagenic metabolites bind to DNA and are capable of inducing G-to-T transversions (2).

In certain areas of the HCC endemic regions, an unusual mutational hot spot has been reported in the p53 tumor suppressor gene (3–8). This mutation, at the third base of codon 249 in exon 7, is an AGG-to-AGT transversion (arginine to serine). HCCs from other areas of the world have been reported to have markedly lower frequencies of codon 249 mutations (9–16). The geographic distribution of these mutations and the similarity to the transversions produced *in vitro* by AFB_1 suggested to investigators that exposure to AFB_1 caused the mutations (3, 4). Recent demonstration of the preferential mutability of codon 249 by rat liver microsome-activated AFB_1 in HepG2 cells has supported the hypothesis (17).

While rates of chronic HBV infection and AFB_1 exposure are both elevated in HCC endemic areas, HCC rates in those regions of Asia are an order of magnitude higher than are the rates in areas of Africa with comparable exposures (18). A possible explanation for the difference in rates may be that individuals in these populations vary in their capacity to detoxify the mutagenic metabolite of AFB_1, aflatoxin 8,9-epoxide. Microsomal epoxide hydrolase (EPHX) and glutathione *S*-transferase M1 (GSTM1) are both involved in AFB_1 detoxification in hepatocytes. EPHX detoxifies the epoxide by conversion into 1,2-dihydrodiols and GSTM1 conjugates the epoxide to glutathione (19, 20). Therefore, lack or diminution of these enzymes might leave more of the epoxide available to bind to DNA. The GSTM1 isozyme is known to be polymorphic in humans as a consequence of a gene deletion (21). It has been demonstrated that individuals who lack the gene also lack GSTM1 enzymatic activity (22). EPHX activity varies within populations and several DNA polymorphisms have been described (23). Recently, a correlation between mutant alleles of EPHX and diminished activity has been reported by Hassett and colleagues (24). The authors demonstrated, in *in vitro* expression studies of cDNA, that substitution of His-113 for the more commonly occurring Tyr-113 residue in exon 3 decreased EPHX activity ≈40%.

To test whether EPHX and GSTM1 genotypes were associated with serum AFB_1-albumin adducts, HCC, and p53 codon 249 mutations, studies were conducted in the HCC endemic areas of Ghana and China. The hypotheses tested were that mutant alleles at one or both loci would be associated with increased levels of serum AFB_1-albumin adducts, with HCC and with p53 codon 249 mutations.

MATERIALS AND METHODS

Study Participants. *Ghana.* To test whether GSTM1 or EPHX genotypes were related to the presence of serum AFB_1-albumin adducts, a cross-sectional study was conducted in Ghana. Peripheral blood samples were obtained from 49 unrelated adult Ghanaian males. The presence of serum AFB_1-albumin adducts reflects short-term exposure to AFB_1 and is, presumably, predictive of DNA damage (25). All Ghanaian participants were healthy gold miners employed by the Ashanti Goldfields Corporation in Obuasi, Ghana. Blood samples obtained in Obuasi were separated into components and sent on dry ice to Philadelphia.

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Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; AFB_1, aflatoxin B_1; EPHX, epoxide hydrolase; GSTM1, glutathione *S*-transferase M1; HBsAg, HBV surface antigen; OR, odds ratio.

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China. To study whether EPHX and GSTM1 genotypes were related to HCC and to p53 codon 249 mutations, a case–control study was conducted. The cases were 52 individuals with HCC and the controls were 116 healthy individuals. The individuals with HCC were a subset of the HCC patients treated at the Zhong Shan Hospital in Shanghai between 1991 and 1992. The majority of the patients at the Zhong Shan Hospital reside in Shanghai and the surrounding geographical area. Tumor and normal tissue samples were obtained at the time of resection. Data were abstracted from the medical records of participants concerning the date of diagnosis, the individual's HBV marker status, including HBV surface antigen (HBsAg), and the individual's sex and age at diagnosis. Forty-three of the 52 HCC patients were male (83%) and 9 were female. Seventy-seven percent (37/48) of the cases tested were positive for HBsAg. The cases ranged in age from 30 to 70 years. The 116 healthy controls were residents of Haimen City, Jiangsu Province, which is located on the opposite bank of the Yangtze River, ≈10 km from Shanghai. Haimen City is within the catchment area of the Zhong Shan Hospital. The control individuals are members of an ongoing cohort study of HCC in Haimen City and ranged in age from 19 to 67 years. Eighty-one of the control participants were male (70%). All individuals were chosen from separate villages in Haimen City in an effort to select unrelated persons. All members of the cohort were tested for HBsAg as part of the cohort study. Constitutional DNA was obtained from each participant by spotting peripheral blood onto filter cards.

Case and control participants were genotyped for EPHX and GSTM1. Case participants were typed for p53 codon 249 mutations as well in both their tumor and normal tissues.

DNA Analysis. DNA was extracted from filter cards of control participants from China by the procedure described by McCabe (26). Briefly, a spot of blood was punched into an Eppendorf tube, which was autoclaved. Four hundred microliters of 10 mM Tris·HCl, pH 8.3/50 mM KCl/2.5 mM $MgCl_2$/0.1% Triton X-100/0.1% gelatin was added. The tube was boiled and spun, and the supernatant was decanted. DNA was extracted from the tissue of Chinese patients by standard techniques (27). DNAs were amplified on a thermal cycler (MJ Research, Cambridge, MA). Fifteen nanograms of DNA was amplified in a total vol of 5 μl containing 10 mM Tris·HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 10 μM each deoxynucleotide triphosphate, 0.5 μM each primer, and 0.25 unit of *Taq* polymerase (Perkin–Elmer) overlaid with two drops of mineral oil. GSTM1 genotyping was performed with the primers described by Comstock *et al.* (28) and Brockmöller *et al.* (22), which discriminate homozygous deletion of the locus (null) from hemizygous or homozygous presence. EPHX genotyping was performed with a single strand conformation polymorphism assay that detects a biallelic polymorphism at nucleotide 499 (residue 113; exon 3) (24, 29) in the previously published cDNA sequence (23). All assays were repeated to confirm genotype and a control PCR was performed on each sample to verify that the DNA template was capable of PCR amplification. p53 codon 249 analysis was performed by the previously published restriction endonuclease assay (4).

Serum Analysis. Serum AFB_1-albumin adducts were measured by an ELISA as described by Chapot and Wild (30). Serum levels of <5 pg/mg were deemed to be undetectable. HBsAg status was determined by the third generation assay of Abbott (Auszyme Monoclonal).

Statistical Analysis. The relationship between EPHX and GSTM1 genotypes and serum AFB_1-albumin adducts was assessed by one-way analyses of variance and χ^2 analyses once the adduct levels were dichotomized. The relationship between genotypes and the presence or absence of HCC was determined by χ^2 analyses. Log-linear modeling was done to account simultaneously for the effect of genotype and HBsAg status on disease state. The relationships among genotype, HBV status, and p53 mutation status were determined by χ^2 analyses and log-linear modeling. Odds ratios (ORs) (the ratio of the odds in favor of the presence of a factor among the cases to the odds in favor of the presence of a factor among the controls) were calculated to determine the strength of the relationships described.

Table 1. AFB_1-albumin adduct distribution by EPHX and GSTM1 genotype in Ghanaian males

	EPHX genotype			GSTM1 genotype		
AFB_1	1/1	1/2 or 2/2	Total	Present	Null	Total
≤5 pg/mg	21	4	25	19	6	25
>5 pg/mg	13	11	24	11	13	24
Total	34*	15*	49	30†	19†	49

*$\chi^2 = 5.1$; $P = 0.02$.
†$\chi^2 = 4.7$; $P = 0.03$.

Table 2. HCC cases and controls by GSTM1 genotype in China

	GSTM1		
	Present	Null	Total
Cases	23	29 (56%)	52 (100%)
Controls	69	47 (41%)	116 (100%)
Total	92*	76*	168

*$\chi^2 = 3.4$; $P = 0.06$; OR (95% confidence interval) = 1.9 (0.94–3.63).

RESULTS

AFB_1/Genotype Study. The results of the AFB_1/genotype study showed that the men with detectable AFB_1-albumin adduct levels (>5 pg/mg) were more likely to have either an EPHX 1/2 or 2/2 genotype ($\chi^2 = 5.1$; $P = 0.02$) than were men without detectable levels. Similarly, men who were GSTM1 null ($\chi^2 = 4.7$; $P = 0.03$) were more likely to have detectable AFB_1-albumin adduct levels than were men who were not GSTM1 null. When the genotypes were combined, 100% of the men with both high-risk genotypes (at least one EPHX- /2 allele; GSTM1 null) had detectable levels of AFB_1-albumin adducts, in contrast with only 32% of the men with both low-risk genotypes (EH 1/1; GSTM1 not null) (Table 1). One-way analyses of variance determined that the mean AFB_1-albumin adduct levels were higher in the men with at least one mutant EPHX allele (11.5 vs. 7.08 pg/mg) and in men who were GSTM1 null (9.8 vs. 7.5 pg/mg), although neither difference attained statistical significance.

Three of the 49 Ghanaian participants (6%) were positive for HBsAg. The small number in the $HBsAg^+$ group precluded meaningful analysis after stratification on HBsAg status.

HCC/Genotype Study. In accord with the results of the AFB_1/genotype study, the frequency of the GSTM1 null genotype was greater among the cases (56%) than the controls (41%) and the difference was statistically significant at $P = 0.047$ in a one-tailed test (Table 2). The difference in EPHX distributions between the HCC cases and controls was highly significant (Table 3) and remained so after adjustment for multiple comparisons ($\chi^2 = 8.7$; $P = 0.01$). As hypothesized, the mutant EPHX allele, allele 2, was significantly overrepresented among the cases. The OR associated with having at

Table 3. HCC cases and controls by EPHX genotype in China

	EPHX genotype			
	1/1	1/2	2/2	Total
Cases	5 (10%)	18	29	52 (100%)
Controls	30 (25%)	46	40	116 (100%)
Total	35*	64*	69*	168

*$\chi^2 = 8.7$; $P = 0.01$; OR (95% confidence interval) = 3.3 (1.2–9.2).

Table 4. Relationship of HBsAg positivity and EPHX genotype on risk of HCC

	HBsAg$^-$			HBsAg$^+$		
EPHX	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)
1/1	1	25	1.0	3	5	15.00 (1.2, 184)
1/2	1	40	3.3 (0.39, 28.6)	15	6	77.27 (8.9, 665.8)
2/2	9	35		19	5	

least one mutant EPHX allele was 3.3, while the OR associated with being GSTM1 null was 1.9. The OR associated with having at least one mutant EPHX allele and being GSTM1 null was 9.4.

Sixteen of the control participants were positive for HBsAg (13.8%), in contrast with 37 of the 48 tested case participants (77%). As expected, the association between HBsAg$^+$ and HCC case status was highly significant ($\chi^2 = 62$; $P < 0.0001$; OR = 21.0). Given that both HBsAg status and EPHX genotype were related to HCC risk, ORs were calculated for the risk of HCC, given combinations of the two factors (Table 4). In contrast with individuals with neither high-risk factor (i.e., HBsAg$^-$/EPHX 1/1), individuals with just the high-risk genotype (i.e., HBsAg$^-$/at least one EPHX- /2 allele) had >3 times the risk of HCC and individuals with just the viral infection (i.e., HBsAg$^+$/EPHX 1/1) had 15 times the risk of HCC. Individuals, however, with both the viral infection and the high-risk genotype had >77 times the risk of HCC of individuals with neither factor. This synergism of the two factors could be explained by an examination of the data in Table 4. Among the HBsAg$^-$ individuals, risk of HCC was not increased until two copies of the EPHX mutant allele were present. Among the HBsAg$^+$ individuals, however, risk of HCC increased in the presence of only one EPHX mutant allele.

Given the synergism of HBV and EPHX genotype in producing risk, an examination of age at diagnosis was done to determine whether individuals with multiple risk factors were likely to develop disease earlier than individuals with fewer factors. Individuals of the highest-risk status, HBsAg$^+$ and EPHX 2/2 genotype, had a mean age at diagnosis of 48 years. In contrast, HBsAg$^+$ individuals with only one mutant EPHX allele and HBsAg$^-$ individuals with an EPHX 2/2 genotype had mean ages at diagnosis of 52 years.

p53 codon 249 mutations were detected in 10 of the 52 tumor samples (19%) while nontumor DNA from all 10 individuals showed a p53 wild-type genotype. All 10 of the mutations occurred in tumors of individuals who had an EPHX mutant allele ($\chi^2 = 1.3$; $P = 0.25$) and 8 of the 10 occurred in individuals who were GSTM1 null ($\chi^2 = 2.0$; $P = 0.08$). No mutations occurred in individuals with two low-risk genotypes (Table 5). Nine of the 10 individuals with p53 codon 249 mutations had been typed for HBsAg and all were HBsAg$^+$ ($\chi^2 = 3.2$; $P = 0.07$). There were no differences in p53 mutation rate by sex or age of the case.

To determine whether there were differences in genotype distribution by country, the EPHX and GSTM1 distributions were compared in the Ghanaian participants and Chinese controls. While there was no difference in the GSTM1 null frequencies in the two groups (Table 6), the EPHX distributions were significantly different (Table 7). Sixty-nine percent of the Ghanaian participants had the low-risk EPHX 1/1 genotype in contrast with only 26% of the Chinese control participants ($P < 0.0001$).

Table 5. p53 codon 249 mutation status by EPHX/GSTM1 genotype in HCC cases in China

	Genotype			
p53	EH↓/GST↓	Intermediate	EH↑/GST↑	Total
Mutant	0	2	8	10
Wild type	1	24	17	42
Total	1	26*	25*	52

↓, low risk; ↑, high risk.
*$\chi^2 = 5.1$; $P = 0.07$.

DISCUSSION

In this paper, we report a significant association between EPHX and GSTM1 genotypes and the presence of AFB_1 adducts. We also report a significant association between EPHX genotype and HCC. The association between GSTM1 genotype and HCC is suggestive, as are the relationships between the genotypes and p53 codon 249 mutations.

An association among detoxification genotypes, AFB_1-albumin adducts, and HCC has biologic plausibility. The AFB_1 parent compound is not harmful prior to metabolic activation to form AFB_1–8,9-epoxide via the phase I detoxification pathway. The epoxide can then be rendered innocuous via phase II detoxification, in which GSTM1 conjugates the epoxide to glutathione and EPHX converts the epoxide into AFB_1-dihydrodiol (19, 20). Alternatively, the epoxide can bind to DNA at the N^7 guanine residue. The lack or diminution of the phase II detoxification enzymes would then leave more of the epoxide available to bind to DNA. Therefore, it could be hypothesized that persons who had an impaired ability to detoxify the mutagenic AFB_1 metabolite would be more likely to suffer the related effects. Similarly, the association between detoxification genotypes and p53 codon 249 mutations is not entirely unexpected in that GSTM1 and EPHX enzymes remove the AFB_1 epoxide speculated to cause the mutations in p53 at codon 249.

The failure to detect a statistically significant relationship between GSTM1 genotype and case/control status may be due to one of several reasons. The most obvious explanation is that a significant association may not exist. The existence of the AFB_1/GSTM1 association, however, is some evidence that GSTM1 may be important in AFB_1-related HCC. Alternatively, our failure to detect an association may simply be due to an inadequate sample size given that the *P* value approached significance and the GSTM1 null type can be considered a very

Table 6. Comparison of GSTM1 genotype distributions by country

	GSTM1		
Country	Present	Null	Total
Ghana	30	19 (39%)	49 (100%)
China	69	47 (41%)	116 (100%)
Total	99*	66*	165

*$\chi^2 = 0.04$; $P = 0.83$.

Table 7. Comparison of EPHX genotype distributions by country

	EPHX genotype			
Country	1/1	1/2	2/2	Total
Ghana	34 (69%)	11	4	49 (100%)
China	30 (26%)	46	40	116 (100%)
Total	64*	57*	44*	165

*$\chi^2 = 28.7$; $P = < 0.0001$.

common exposure. In fact, given the genotype frequencies observed, the study had only 58% power to detect a difference at a 0.05 significance level. Roughly doubling the sample size (100 cases and 200 controls) would result in 80% power to detect a difference between cases and controls at the 0.05 significance level. Finally, it may be that GSTM1 plays a less significant role in detoxification of other environmental exposures than EPHX. As demonstrated in the Ghana/China genotype comparisons, the GSTM1 frequencies are quite similar, while the EPHX distributions are markedly discrepant, suggesting that EPHX may have more to do with the differing HCC rates than GSTM1. The role of GSTM1 in HCC, however, certainly bears further investigation.

The effect of chronic hepatitis B infection on HCC risk is well described. Less well understood are the factors that distinguish those HBV carriers who develop HCC from the HBV carriers who do not. Based on data from a large prospective study (31, 32), it has been suggested that AFB_1 exposure may increase risk in HBV carriers. Based on the determination of urinary AFB_1 biomarkers, the authors reported relative risks of 3.4 in $HBsAg^-/AFB_1^+$ persons, 7.3 in $HBsAg^+/AFB_1^-$ persons, and 59.4 in $HBsAg^+/AFB_1^+$ persons. These risk estimates are consistent with the ORs in our data in which $HBsAg^-$ persons with at least one EPHX mutant allele had a 3.3-fold increased risk, $HBsAg^+$/EPHX 1/1 persons had a 15-fold increased risk, and $HBsAg^+$ individuals with at least one EPHX mutant allele had a 77-fold increased risk. Also in accord with the results of the prospective study were the estimates of risk attributable to AFB_1 exposure in the prospective study and EPHX genotype in our study. The prospective study data indicate an AFB_1 attributable risk of 52%, where our data indicate an estimated EPHX attributable risk of 59%. The similarity of these estimates may indicate that in the presence of widespread AFB_1 exposure, the two indices are measuring, essentially, the same risk variable. An additional finding of the prospective study, that the presence of AFB_1 urinary metabolites did not correlate with reported dietary intake or with measured AFB_1 in foodstuffs, supports the importance of AFB_1 metabolism in determining risk.

Our results indicate that constitutional genotypes at certain detoxification loci may be significant risk factors for HCC. Given that the control Chinese population had a significantly greater number of individuals with a high-risk genotype, but not a significantly greater number of $HBsAg^+$ individuals than the Ghanaian population, these data support the hypotheses that genotype differences may explain HCC rate differences in the two areas. In particular, the difference in the EPHX distributions may be of particular significance.

Genetic susceptibility, in humans, due to the inheritance of a mutation in a tumor suppressor gene, is now well established (33). From results of this study, we suggest that genetic susceptibility may also occur through an alternative mechanism: inheritance of a genotype that increases the probability of mutations occurring at etiologic loci such as p53. Furthermore, this susceptibility appears to occur in conjunction with exposure to an environmental carcinogen. The identification of an EPHX/GSTM1–AFB_1–p53 link in HCC suggests that genetic susceptibilities to the effects of other carcinogens occur among human populations and may be involved in the etiology of common cancers.

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