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Chronic Hepatitis B Carriers with Null Genotypes of Glutathione S-Transferase M1 and T1 Polymorphisms Who Are Exposed to Aflatoxin Are at Increased Risk of Hepatocellular Carcinoma

Chien-Jen Chen,[1] Ming-Whei Yu,[1] Yun-Fan Liaw,[2] Lian-Wen Wang,[3] Sinnabhatr Chiamprasert,[3] Farhan Matin,[3] Ari Hirvonen,[4] Douglas A. Bell,[4] and Regina M. Santella[3]

[1]Institute of Epidemiology, College of Public Health, National Taiwan University, and [2]Liver Unit, Chang-Gung Memorial Hospital, Taipei; [3]Division of Environmental Health Sciences, School of Public Health, Columbia University, New York; and [4]Laboratory of Biochemical Risk Analysis, National Institute of Environmental Health Sciences, Research Triangle Park

Summary

This study was carried out to elucidate the effect of glutathione S-transferase (GST) M1 and T1 polymorphisms on the aflatoxin-related hepatocarcinogenesis among chronic carriers of hepatitis B surface antigen (HBsAg). A total of 32 newly diagnosed hepatocellular carcinoma (HCC) cases and 73 age-matched controls selected from a cohort of 4,841 chronic HBsAg carriers who had been followed for 5 years were studied. The level of aflatoxin B_1 (AFB_1)-albumin adducts in their serum samples collected at the recruitment was examined by competitive enzyme-linked immunosorbance assay, and genotypes of GST M1 and T1 were determined by PCR. There was a dose-response relationship between serum level of AFB_1-albumin adducts and risk of HCC. The biological gradients between serum AFB_1-albumin adducts level and HCC risk were observed among chronic HBsAg carriers who had null genotypes of GST M1 and/or T1 but not among those who had non-null genotypes. The multivariate-adjusted odds ratios of developing HCC for those who had low and high serum levels of AFB_1-albumin adducts compared with those who had a undetectable adduct level as the referent (odds ratio = 1.0) were 4.1 and 12.4, respectively, for HBsAg carriers with null GST M1 genotype ($P < .01$, on the basis of the significance test for trend); 0.7 and 1.4 for those with non-null GST M1 genotype ($P = .98$); 1.8 and 10.2 for those with null GST T1 genotype ($P < .05$); and 1.3 and 0.8 for those with non-null GST T1 genotype ($P = .93$). The interaction between serum AFB_1-albumin adduct level and polymorphisms of GST M1 and T1 was at marginal statistical significance levels ($.05 < P < .10$).

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Address for correspondence and reprints: Dr. Chien-Jen Chen, Institute of Epidemiology, College of Public Health, National Taiwan University, 1 Jen-Ai Road Section 1, Taipei 10018, Taiwan. E-mail: cjchen@ntumc1.mc.ntu.edu.tw

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Introduction

Hepatocellular carcinoma (HCC) is a highly malignant disease with an extremely poor prognosis. It is a major cancer, with 1,000,000 deaths annually in the world (Bosch and Munoz 1989). Both viral and chemical carcinogens are involved in development of HCC in humans, and chronic hepatitis B virus (HBV) infection is the most important risk factor for HCC in Taiwan as in other countries (Yu and Chen 1994). There are almost 300,000,000 chronic hepatitis B carriers in the world, with the highest prevalence in Southeast Asia, sub-Saharan Africa, and Greenland (Tiollais et al. 1985). About one-fifth of chronic carriers are expected to develop HCC in their lifetime (Beasley 1988). The fact that HCC is not an inevitable consequence of chronic HBV infection has stimulated the search for other HCC risk factors. In addition to chronic carrier status of HBV surface antigen (HBsAg) and e antigen, cigarette smoking, habitual alcohol consumption, seropositivity of antibodies against hepatitis C virus (anti-HCV), elevated serum testosterone level, low vegetable consumption frequency and decreased serum retinol level, and aflatoxin exposure have been documented as risk factors for HCC in Taiwan (Chen et al. 1991, 1993; Lin et al. 1991; Yu et al. 1991, 1995; Hatch et al. 1993; Yu and Chen 1993; Chang et al. 1994).

Aflatoxin B_1 (AFB_1) is the most potent hepatocarcinogen in a variety of animal species (Dragan and Pitot 1994). It is metabolized by the microsomal mixed-function oxygenase enzyme system to various reduced and oxidized derivatives, including an unstable reactive AFB_1-8,9-epoxide, which can bind covalently to nucleophilic sites of biological macromolecules, including nucleic acids and proteins (Gallagher et al. 1994). The formation of AFB_1-guanine adducts through interaction between AFB_1-8,9-epoxide and hepatic DNA has been shown to be critical for the carcinogenesis induced by AFB_1 in animals (Kensler et al. 1986). AFB_1-guanine adducts are lost rapidly from DNA and excreted in the urine of AFB_1-treated animals.

A significant ecological correlation between aflatoxin

exposure and human HCC has been reported in Taiwan and other countries (Shank et al. 1972; Peers and Linsell 1973; Peers et al. 1976, 1987; Van Rosenburg et al. 1985; Yeh et al. 1989; Allen et al. 1992; Hatch et al. 1993). However, valid estimation of internal dose and biologically effective dose for individual exposure to aflatoxin is still being developed. On the basis of an indirect immunofluorescence method (Zhang et al. 1991), AFB_1-DNA adducts were detectable in liver tissues from 49 (64%) of 77 HCC patients in Taiwan (Zhang et al. 1991; Chen et al. 1992). A synergistic effect on HCC has recently been observed between urinary level of aflatoxins and HBsAg carrier status in Shanghai, China (Ross et al. 1992).

Because urinary aflatoxin level reflects intake on the previous day, it is an excellent marker for short-term exposure, but it may not reflect long-term intake by individuals. While AFB_1-guanine is the major DNA adduct, AFB_1-albumin is the major protein adduct found in peripheral blood. Their use as a biomarker for aflatoxin exposure has several advantages: (1) aflatoxin-albumin adducts reflect DNA damage in hepatocytes, as does aflatoxin-N^7-guanine in urine (Wild et al. 1986); (2) albumin adducts, at least in experimental animals, are as long-lived as albumin, which has a half-life of 21 d in humans, and thus provide a measure of exposure over a period of 2–3 mo (Sabbioni et al. 1987); and (3) multiple measurements of urinary aflatoxin are required to reflect average exposure, but only a single measurement of albumin adducts is needed to provide a representative average exposure (Hall and Wild 1994). In other words, serum level of AFB_1-albumin adducts is a better estimate of long-term biologically effective dose of aflatoxin exposure than is urinary level of aflatoxin-N^7-guanine. However, the association between serum AFB_1-albumin adducts content and HCC risk at an individual level has never been reported.

Glutathione S-transferases (GSTs) are a unique group of multifunctional isozymes that play an important role in the conjugation and detoxification of various xenobiotics, such as aflatoxins and polycyclic aromatic hydrocarbons (Liu et al. 1991; Bell et al. 1992). GST M1 and T1 are polymorphic in humans, and deficiency in their enzyme activity is caused by the inherited homozygous absence of the genes (Strange 1993; Pemble et al. 1994). The proportion of GST M1 null genotype was reported to increase in lung and bladder cancer patients compared with controls (Zhong et al. 1991; Bell et al. 1993; Strange 1993). GST M1 plays an important role in detoxifying DNA reactive metabolites of AFB_1 (Liu et al. 1991), but the effect of GST T1 on aflatoxin detoxification remains unclear. It has been shown that the 100,000-fold difference between mice and rats in liver cancer response to doses of AFB_1 is attributable to the difference in GST-mu activity between species, and the resultant difference in detoxification of the AFB_1 epoxide, which is formed just as readily in mice as in rats (Eaton and Gallagher 1994). Whether the aflatoxin-related HCC risk is also modified by genotypes of GST M1 and T1 in humans remains to be elucidated.

The specific aim of this study is to assess the effect of genotypes of GST M1 and T1 on the AFB_1-related hepatocarcinogenicity in chronic HBsAg carriers. A dose-response relationship was observed between serum level of AFB_1-albumin adducts and risk of HCC. The biological gradient was observed for chronic HBsAg carriers who had null genotypes of GST M1 and/or T1, but not for those who had non-null genotypes at all.

Subjects and Methods

Study Subjects

A cohort of 4,841 male, asymptomatic, chronic HBsAg carriers aged from 30 to 65 years was recruited from the Government Employee Central Clinics and the Liver Unit of Chang-Gung Memorial Hospital in Taiwan from August 1988 to June 1992. They all gave their consent to participate in this study on a voluntary basis. At recruitment, each study subject was personally interviewed according to a structured questionnaire, to obtain information on demographic characteristics, habits of cigarette smoking and alcohol drinking, dietary consumption frequency, and personal and family history of various chronic diseases. Both duration and quantity of cigarette smoking and alcohol drinking were queried. "Having ever smoked cigarettes" was defined as having smoked cigarettes $\geq$4 d/wk for $\geq$6 mo, "having ever drunk alcohol" as having consumed alcoholic beverage $\geq$1 d/wk for $\geq$6 mo. Questionnaire interview was carried out by public health nurses who were well trained to standardize their interview techniques. Blood specimens, including serum and white blood cells from study subjects, were collected, separated, and stored at -70°C until subsequent analysis. HBsAg and anti-HCV were tested, respectively, by radioimmunoassay and enzyme immunoassay using commercial kits (Abbott).

Follow-up of study subjects was performed through various channels, including annual health examination, personal telephone interviews, abstraction of medical records, and data linkage with national death certification and cancer registry systems. The diagnosis of HCC was based on (1) positive findings on cytological or pathological examination and/or (2) positive images on angiogram, ultrasonography, and/or computerized tomography, combined with an alpha-fetoprotein level >400 ng/ml. There were 37 new HCC cases identified during the follow-up period. Controls were selected from the cohort of HBsAg carriers who remained unaffected throughout the period. They were matched with cases on the basis of age, recruitment clinic, and date

of biospecimen collection (within 3 mo) and randomly selected within matching strata. Because there were five HCC cases and three matched controls who had no adequate biospecimens for the determination of serum AFB_1-albumin adducts level and/or genotypes of GST, a total of 32 HCC cases and 73 matched controls were included in this study.

Serum AFB_1-Albumin Adducts Level

Albumin was prepared from plasma essentially as described by Wild et al. (1990*a*), and concentration was determined with bicinchoninic acid (BCA Reagent Pierce). For the digestion, 2 mg albumin and 0.5 mg proteinase K were incubated 15 h at 37°C. Adducts were isolated by the procedure of Sheabar et al. (1993), dissolved in 0.5 ml phosphate buffer solution containing 1% FCS and 1 mM phenylmethyl-sulfonylfluoride, to inhibit residual protease activity, and assayed by competitive enzyme-linked immunosorbance assay (ELISA) using a polyclonal antiserum. The detection limit of serum AFB_1-albumin adducts level was 0.01 fmol/μg.

In order to prepare an antiserum for the detection of AFB_1-albumin adducts, human serum albumin and bovine gamma globulin were modified with AFB_1 epoxide synthesized by the method of Baertschi et al. (1988). Human serum albumin or bovine gamma globulin (9 mg in 5 ml 0.05 M phosphate buffer pH 7.4) were mixed with 0.2 mg of the epoxide generated from AFB_1 or [^{3}H] AFB_1 (25 mCi/mmol; Moravic) in methylene chloride and incubated overnight at room temperature. The samples were dialyzed extensively to remove nonbound material. Modification levels were determined from the absorbance at 450 nm (e = 12,700) or the specific activity and ranged from 0.6 to 2.3 mol AFB_1/mol protein. New Zealand white rabbits were immunized by intramuscular injection at four sites with 1 mg of the modified bovine gamma globulin (2.3 mol/mol) in complete Freund's adjuvant, followed by monthly boosting with 1 mg protein in incomplete adjuvant. Antiserum (#7) was characterized by competitive ELISA. For the ELISA standard curve, [^{3}H] AFB_1-human serum albumin was digested with proteinase K as described above for the human samples and adducts isolated by Seppak C18 (Waters) extraction using cartridges that had been prewashed with 10 ml chloroform, 10 ml methanol, and 10 ml water. After application of the sample in phosphate buffer and washing with 10 ml water and 5 ml 5% methanol, the adducts were isolated with 5 ml 80% methanol. Adducts levels were determined from the specific activity.

Ninety-six microwell plates (Easywash, Corning) were coated with 3 ng AFB_1-human serum albumin by drying phosphate buffer solution. Nonspecific binding was blocked by incubation for 1 h at 37°C with 1% FCS in phosphate buffer solution containing 0.05% Tween 20. Standards (50 μl containing 3–100 fmol adduct) or human samples (equivalent of 100 μg) were then added to each well, followed by the antiserum (50 μl 1:200,000 dilution) and the plates incubated 90 min at 37°C. After washing, goat anti-rabbit IgG akaline phosphatase (1:500 dilution, Boehringer Mannheim) was added and the plates incubated at 37°C for 90 min. After washing, 0.1 ml 1 mg/ml p-nitrophenyl phosphate in 1 M diethanolamine (pH 8.6) was added to each well. The absorbance at 405 nm was measured on a Dynatech MR 2000 microplate reader (Dynatech Laboratories). This assay had 50% inhibition of antiserum binding at 10–20 fmol AFB_1-adduct per well. The limit of sensitivity (20% inhibition), when assaying the equivalent of 200 μg albumin/well, was 0.01 fmol/μg. Samples were assayed by duplicate analysis in duplicate wells; samples with <20% inhibition were considered undetectable. Two control samples were analyzed with each batch of sera, a pooled sample of plasma from nonsmoking U.S. subjects, and a positive control of serum from a rat treated with 1.5 mg AFB_1.

Genotypes of GST M1 and T1

Genotype of GST M1 was identified in leukocyte DNA after PCR amplification with primers to exons 6 and 7, which produced a 210-bp band (Bell et al. 1993), while GST T1 genotype was determined by using the technique of Pemble et al. (1994) with the additional modification that beta-globin primers were added to the PCR (Bell et al. 1993).

Statistical Analysis

Because it was not considered appropriate to assign a value to the undetectable serum level of AFB_1-albumin adducts, the adducts level was analyzed as a categorical, rather than continuous, variable. It was categorized into three groups (undetectable, low detectable, and high detectable), to examine the dose-response relationship between aflatoxin exposure and HCC risk. The serum level of AFB_1-albumin adducts between HCC cases and matched controls was first compared. Odds ratios with 95% confidence intervals were calculated to indicate the magnitude of associations between serum level of AFB_1-albumin adducts and HCC risk. Mantel-Haenszel χ^2 tests for trend were used to examine the statistical significance of the biological gradient. Multiple logistic regression analysis was used to estimate odds ratios of developing HCC for serum AFB_1-albumin adducts level after adjustment for habits of cigarette smoking and alcohol drinking, which have been documented as risk factors for HCC (Chen et al. 1991, 1993). The dose-response relationships between aflatoxin and HCC were further examined through stratification analyses for different groups categorized by genotypes of GST M1 and T1. The statistical significance of the interaction be-

Table 1

Serum Level of AFB-Albumin Adducts in Cases Affected with HCC and Matched Controls

Serum AFB-Albumin Level	HCC Cases		Controls		Adjusted Odds Ratio[a] (95% confidence interval)
	No.	(%)	No.	(%)	
Undetectable	14	(43.7)	44	(60.3)	1.0 (referent)
Low	12	(37.5)	24	(32.9)	1.6 (.6–4.0)
High	6	(18.8)	5	(6.8)	3.8 (1.0–14.5)*

[a] Adjustment for habits of cigarette smoking and alcohol consumption through multiple logistic regression analysis.

* *P*-value based on the statistical significance test of odds ratio <.05; *P*-value based on the test for trend = .03.

tween serum AFB_1-albumin adducts level and genotypes of GST M1 and T1 was also examined through logistic regression analysis. SAS/STAT software (SAS Institute Inc.) was used for the data analysis.

Results

Among 32 HCC cases, 12 were diagnosed within 2 years of recruitment and 20 >2 years after recruitment. The means ± SD of age at recruitment for HCC cases and matched controls were 51.3 ± 9.7 and 51.5 ± 9.9 years, respectively. Cases and controls had similar frequency distributions among cases who had ever smoked cigarettes (39% vs. 35%) or drunk alcohol (21% vs. 20%).

Table 1 compares the serum level of AFB_1-albumin adducts between HCC cases and healthy controls. HCC cases had higher levels of serum AFB_1-albumin adducts than matched controls. A statistically significant ($P = .05$, based on Mantel-Haenszel χ^2 test for trend) dose-response relation was observed between serum level of AFB_1-albumin adducts and HCC risk. The higher the serum AFB_1-albumin adducts level, the higher the HCC risk. The biological gradient remained significant ($P = .03$) after adjustment for cigarette smoking and alcohol consumption through multiple logistic regression analysis.

Tables 2 and 3 show the biological gradient between serum level of AFB_1-albumin adducts and risk of HCC stratified by genotypes of GST M1 and T1, respectively. The dose-response relationship between aflatoxin exposure and HCC risk remained statistically significant with increases in the magnitude of odds ratios among chronic HBsAg carriers with null genotypes of GST M1 and T1. However, no associations between aflatoxin and HCC were observed among carriers with non-null genotypes. The monotonic increase in aflatoxin-related HCC risk for chronic HBsAg carriers with null genotypes of GST M1 and T1 remained after adjustment for cigarette smoking and alcohol consumption. The statistical significance for the interaction terms between serum AFB_1-albumin adducts level and genotypes of GST M1 and T1 was marginal, with *P*-values of .06 and .08, respectively, after adjustment for cigarette smoking and alcohol drinking.

Table 2

Biological Gradient Between Serum Level of AFB-Albumin Adducts and Risk of HCC by Genotypes of Glutathione S-Transferase M1

Serum AFB-Albumin Level	Null			Non-Null		
	Case (No.)	Control (No.)	Adjusted Odds Ratio (95% CI)[a]	Case (No.)	Control (No.)	Adjusted Odds Ratio (95% CI)
Undetectable	5	27	1.0 (referent)	9	17	1.0 (referent)
Low	8	14	4.1 (1.0–16.9)*	4	10	.7 (.2–3.2)
High	4	2	12.4 (1.7–92.7)**	2	3	1.4 (.2–10.9)

[a] Adjustment for habits of cigarette smoking and alcohol consumption through multiple logistic regression analysis.

* $P < .05$.

** $P < .01$ (based on the statistical significance test of odds ratio); *P*-value based on the test for trend <.01 for null set and = .98 for non-null set.

Table 3

Biological Gradient between Serum Level of AFB-Albumin Adducts and Risk of HCC, by Genotypes of Glutathione S-Transferase T1[a]

	NULL			NON-NULL		
SERUM AFB_1-ALBUMIN LEVEL	Case (No.)	Control (No.)	Adjusted Odds Ratio (95% CI)[b]	Case (No.)	Control (No.)	Adjusted Odds Ratio (95% CI)
Undetectable	3	19	1.0 (referent)	10	23	1.0 (referent)
Low	4	14	1.8 (.3–9.7)	5	9	1.3 (.3–5.3)
High	5	3	10.2 (1.3–78.2)*	1	2	.8 (.1–12.0)

[a] GSTT1 genotype was not available for four cases and three controls.

[b] Adjustment for habits of cigarette smoking and alcohol consumption through multiple logistic regression analysis.

* $P < .05$ (based on the statistical significance test of odds ratio); P-value based on the test for trend $<.05$ for null set and $=.93$ for non-null set.

Discussion

Aflatoxins are toxic metabolites produced by various *Aspergillus* species. They are common contaminants of human foodstuffs such as maize, corn, peanuts, and rice, especially in sub-Saharan Africa and Southeast Asia (Wild et al. 1990*a*) where both HBV infection and hepatocellular carcinoma are also prevalent. A nested case-control study carried out in Shanghai has shown a synergistic effect on the development of HCC between HBsAg carrier status and urinary aflatoxin biomarkers including unmetabolized aflatoxins, hydroxylated and demethylated metabolites, and aflatoxin-N^7-guanine adducts. This interaction between chemical and viral hepatocarcinogens provides important clues for prevention of HCC among chronic HBsAg carriers. But no dose-response relationship between urinary aflatoxin level and HCC risk was examined in these two reports.

Because urinary aflatoxin level reflects intake on the previous day, it may not reflect long-term intake by individuals unless their exposures to aflatoxin are relatively constant. The serum level of the albumin adducts is a biomarker of choice for biologically effective dose of long-term exposure to aflatoxin. On the basis of such a representative biomarker for average long-term exposure to aflatoxin, we found for the first time a statistically significant dose-response relationship between aflatoxin and HCC, despite the fact that the numbers of cases and controls included in this study were slightly smaller than those in previous studies in Shanghai (Ross et al. 1992; Qian et al. 1994). Since we limited our study on cases and controls who were chronic HBsAg carriers, it is not possible for us to elucidate the interaction between HBsAg carrier status and aflatoxin exposure.

Animal experiment has shown a striking species difference in liver-cancer response to AFB_1, which is readily explained by the species difference in GST-mu activities (Eaton and Gallagher 1994). We consistently found evidence that the aflatoxin-related HCC risk may be modified by genotypes of GST M1 and T1 in humans. The biological gradient between serum AFB_1-albumin adducts level and HCC risk was significant among chronic HBsAg carriers with null genotypes of GST M1 and T1 but not among carriers with non-null genotypes. In other words, those who had no detoxifying enzymes such as GST M1 and T1 are at a considerably greater risk of developing HCC once they are exposed to aflatoxin. The lack of an association in the non-null group could be a consequence of the small sample size. The interaction terms between serum AFB_1-albumin adducts level and genotypes of GST M1 and T1 were at marginal statistical significance levels ($.05 < P < .10$), possibly resulting from the small sample size in this study. However, the observed significant effect on the development of aflatoxin-associated HCC suggests that HBsAg carriers who have a high aflatoxin exposure and no GST M1 and/or T1 enzymes will have a 10-fold increased risk of developing HCC. The gene-environment interaction between aflatoxin exposure and GST polymorphism deserves further elucidation based on a larger sample. The effects of genetic polymorphisms of cytochrome P450 enzymes on the development of HCC should also be investigated.

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