Role of Genetic Polymorphism of Glutathione-*S*-Transferase *T1* and Microsomal Epoxide Hydrolase in Aflatoxin-associated Hepatocellular Carcinoma[1]

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Abstract

Exposure to aflatoxins is a risk factor for hepatocellular carcinoma (HCC). Aflatoxins occur in peanut butter and are metabolized by genetically polymorphic enzymes such as glutathione-*S*-transferases encoded by glutathione-*S*-transferase μ 1 gene (*GSTM1*) and glutathione-*S*-transferase θ 1 gene (*GSTT1*) and microsomal epoxide hydrolase encoded by epoxide hydrolase gene (*EPHX*). The rate at which aflatoxins become activated or detoxified may depend on polymorphisms in the encoding genes. *GSTM1* homozygous deletion was indeed found to modify the association between peanut butter consumption and HCC. In this study, we investigate possible roles of *GSTT1* and *EPHX* polymorphisms in this relationship. From a Sudanese case-control study on HCC, we analyzed data of 112 incident cases and 194 controls. All participants were interviewed using a standardized questionnaire inquiring about social and demographic factors, peanut butter consumption, and other known HCC risk factors. Univariate analysis showed that *GSTT1* polymorphism was not associated with HCC, whereas *EPHX 113HH* and *139HH* genotypes increased the risk of HCC (Odds ratio, 3.10; 95% Confidence interval, 1.18–8.12). Adjustment for age and region of origin slightly attenuated this association (Odds ratio, 2.56; 95% Confidence interval, 0.83–7.95). Interestingly, unlike *GSTM1*, both *GSTT1* and *EPHX* polymorphism did not modify the association between peanut butter consumption and HCC. In conclusion, these epidemiological findings do not suggest significant roles of *GSTT1* and *EPHX* in aflatoxin metabolism, although *EPHX* polymorphism is possibly related to the increased risk of HCC. Further studies are needed to investigate mechanisms by which the *EPHX* polymorphism potentially modifies cancer risk.

Introduction

HCC[3] is one of the major cancer types in developing countries, where most significant risk factors are chronic hepatitis virus infection and exposure to aflatoxins (1). Aflatoxins are produced by *Aspergillus* fungi, which mainly occur in poorly stored maize and peanuts. In Sudan, aflatoxin exposure most likely occurs via consumption of peanut butter (2), which is a popular food. As we reported previously (3), peanut butter consumption is indeed related to the increased risk of HCC in Sudan. Genetic differences in aflatoxin metabolism may explain the observed differences between prevalence rates of HCC in populations with similar aflatoxin exposures and hepatitis infection rates (4).

The most potent mutagenic and carcinogenic of the aflatoxins is AFB1. Fig. 1 depicts the metabolism of AFB1, which is mainly metabolized by cytochrome P450 3A4 into the genotoxic metabolite AFB1–8,9-*exo*-epoxide. This metabolite can bind to DNA, causing G-to-T transversions (5) that may ultimately lead to cancer. Detoxification prevents formation of DNA adducts; the metabolite may be conjugated to glutathione by GSTs or may be hydrolyzed. Hydrolysis occurs spontaneously or is catalyzed by mEH (6, 7).

Two genetically polymorphic GSTs play a role in AFB1 detoxification: GST-μ encoded by the *GSTM1* gene and GST-θ encoded by *GSTT1*. Homozygous deletion of part of these genes (null genotype) results in enzyme deficiency and might therefore lead to hampered detoxification (8). Several studies (9, 10), among which our own Sudanese case-control study (3), showed that of populations exposed to aflatoxins, only subjects carrying the *GSTM1*-null genotype are at increased risk of HCC. After GST-μ, GST-θ showed highest efficiency for conjugation of glutathione to AFB1–8,9-*exo*-epoxide (Refs. 11 and 12; Fig. 1). Homozygous deletion of the *GSTT1* gene was recorded in 24 to 38% of people from African origin (13). One study showed a positive association between *GSTT1*-null genotype and aflatoxin-albumin adduct levels among chronic HBV antigen carriers (10), but this was not confirmed by another study (14).

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[3] The abbreviations used are: HCC, hepatocellular carcinoma; AFB1, aflatoxin B1; mEH, microsomal epoxide hydrolase enzyme; GST, glutathione-*S*-transferase; GST-μ, glutathione-*S*-transferase μ enzyme; *GSTM1*, glutathione-*S*-transferase μ 1 gene; GST-θ, glutathione-*S*-transferase θ enzyme; *GSTT1*, glutathione-*S*-transferase θ 1 gene; *EPHX*, epoxide hydrolase gene; HBV, hepatitis B virus; OR, odds ratio; CI, confidence interval; SE, standard error.

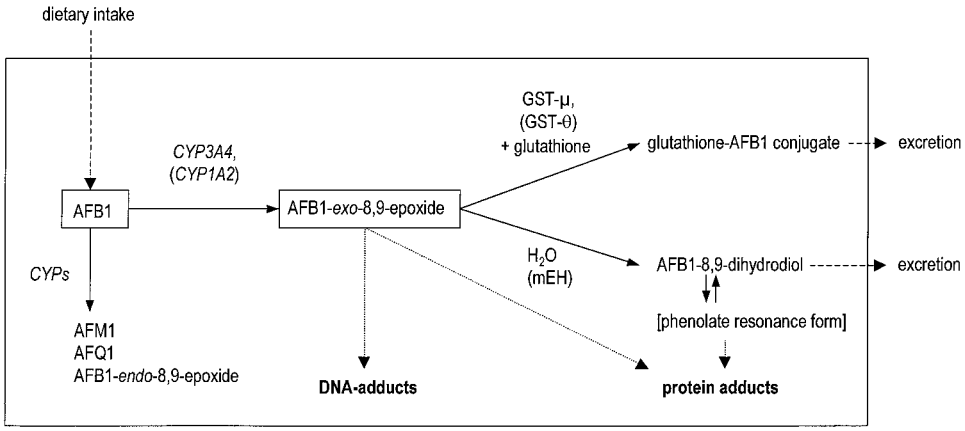

Fig. 1. Proposed metabolism of AFB1. Adapted from Guengerich *et al.* (7) and Eaton *et al.* (6). AFM1, aflatoxin M1; AFQ1, aflatoxin Q1; CYP, cytochrome P450.

The role of mEH in AFB1–8,9-*exo*-epoxide hydrolysis is still unclear because of inconsistent results from biochemical as well as epidemiological studies (11). The encoding *EPHX* gene has two polymorphic sites occurring with allele frequencies of ~30% (15, 16). In exon 3, the *113Y* allele encodes tyrosine incorporation at position 113 of the resulting protein, whereas *113H* codes for histidine. The allelic variants in exon 4, *139H* and *139R*, result in proteins with histidine and arginine at position 139, respectively (15). Both the *113H* and the *139H* allele are related to relatively decreased enzyme expression or stability (15, 17, 18) and may therefore be associated with an increased risk of HCC. Indeed, *EPHX 113HH* genotype increased the risk of HCC in a small case-control study (9) but was not associated to aflatoxin-albumin adduct levels in another study (14).

GSTT1 and *EPHX* polymorphism may especially be significant among subjects carrying the *GSTM1*-null genotype. Few studies (3, 9, 10, 14) addressed the role of *GSTT1* and *EPHX* polymorphism in HCC etiology, and only one (14) simultaneously studied *GSTM1* polymorphism. Moreover, there is some discrepancy between biochemical and epidemiological studies concerning possible roles for GST-μ, GST-θ, and mEH in AFB1 detoxification in ultimate aflatoxin-related HCC etiology. Therefore, we evaluated the possible roles of *GSTT1* and *EPHX* polymorphism and their interaction with aflatoxin exposure (as estimated by peanut butter consumption) in HCC in a Sudanese case-control study, in all subjects and in the subgroup of *GSTM1*-null genotype carriers.

Materials and Methods

Population. A case-control study investigating risk factors for HCC was conducted in Sudan between September 1996 and September 1998. The design and conduct of this study were described in detail by Omer *et al.* (3). In short, subjects residing in West and Central Sudan were eligible. HCC cases were diagnosed clinically by liver function test and ultrasound in five of the six hospitals in the capital Khartoum, situated between West and Central Sudan. Additional diagnosis by liver biopsy and histological examination was available for 95% of cases. All 150 contacted HCC patients provided oral informed consent. ~5% of HCC patients died before they could be invited to participate in the study. Community-based controls were selected randomly in a 1:3 woman:men ratio, because HCC is more prevalent among men and in proportion to the respective population sizes of the two regions. Recruitment was done through sugar shops which hold a complete registration of inhabitants of their serving area. Registration at the shops is required for all inhabitants in the locality, irrespective of their income. In four randomly chosen localities in West Sudan and six localities in Central Sudan, 1 of the 10–15 sugar shops was selected randomly, and 20 households were selected from the sugar shop's registration list. In each household, one control was recruited. All 205 invited subjects agreed to participate.

Data Collection. On the basis of the experience obtained from an explorative study (19), a questionnaire was designed to assess peanut butter intake in a standardized manner. This questionnaire was administered orally by one of the authors (R. E. O.). All participants were interviewed personally, although some cases needed help of an accompanying family member because of serious illness. Cases were interviewed in the hospital, and controls were interviewed at home. Frequency of peanut butter consumption was assessed on a daily or weekly basis and was inquired both "in season" and "off-season," the former corresponding to the period of the year with active agricultural practices, usually June–September. To assess quantity of consumption, we asked if peanut butter is eaten as a part of the meal or as a full meal, and we inquired the amount of peanut butter eaten per meal and the number of persons such a meal is shared with. If a person had stopped to consume peanut butter, the reason for stopping, the time since stopping, and usual peanut butter consumption before stopping were inquired.

Monthly frequency of peanut butter consumption was calculated as a weighted average of the frequencies of peanut butter consumption in season and off season (see Table 1, footnote *c*). The quantity of peanut butter consumed per month was subsequently calculated by multiplication of the average frequency of peanut butter consumption by the amount of peanut butter consumed per meal per person. The questionnaire also identified other HCC risk factors, such as hepatitis infection, smoking, and alcohol consumption.

For determination of genotypes and chronic hepatitis infection, blood samples were collected in 10-ml Venoject tubes. Blood samples of cases were drawn at the hospital, centrifuged immediately, and subsequently stored at −20°C. Because of severe illness, it was not possible to obtain a blood sample of 37 cases (25% of cases). Controls were sampled at home, and blood samples were first transferred in coolers at 4°C to regional hospital laboratories where they were centrifuged and then transferred to Khartoum National Health Laboratory. Here, all samples were kept at −20°C until transport to Wageningen, the Netherlands for further analysis. Of six controls (3%), no blood was available because of logistic difficul-

Table 1 General characteristics of the study population

	Total population		West Sudanese population		Central Sudanese population	
	Cases (n = 112)	Controls (n = 194)	Cases (n = 71)	Controls (n = 80)	Cases (n = 41)	Controls (n = 114)
Demographics						
Region, *n* from West Sudan (%)	71 (63.4)	80 (41.2)[a]	NA[b]	NA	NA	NA
Age, mean (SD)	57.0 (12.2)	44.9 (10.9)[a]	56.9 (11.7)	48.2 (11.4)[a]	56.9 (13.1)	42.5 (10.0)[a]
Gender, *n* males (%)	86 (76.8)	146 (75.3)	56 (78.9)	60 (75.0)	29 (72.5)	86 (75.4)
Education, *n* illiterate (%)	78 (69.6)	67 (34.5)[a]	50 (70.4)	38 (47.5)[a]	27 (67.5)	29 (25.4)[a]
Peanut butter consumption, mean (SD)						
Average frequency (times/month)[c]	11.3 (11.8)	7.9 (9.1)[a]	12.7 (13.6)	7.2 (8.5)[a]	9.1 (7.4)	8.4 (9.5)
Average consumption (kg/month)[c]	0.68 (1.1)	0.29 (0.6)[a]	0.83 (1.22)	0.28 (0.47)[a]	0.44 (0.77)	0.30 (0.63)
Other risk factors, *n* (%)						
Hepatitis B infection[d]	45 (41.3)	14 (7.3)[a]	31 (44.9)	7 (8.8)[a]	14 (35.0)	7 (6.2)[a]
Hepatitis C infection[d]	13 (11.9)	3 (1.6)[a]	6 (8.7)	1 (1.3)[a]	7 (17.5)	2 (1.8)[a]
Positive history of alcohol consumption	44 (39.3)	60 (30.9)	29 (40.9)	32 (40.0)	15 (37.5)	28 (24.6)
Positive history of smoking	45 (40.2)	76 (39.2)	27 (38.0)	33 (41.3)	18 (45.0)	43 (37.7)
Genotype, *n* (%)						
GST[e]						
GSTM1 null	47 (42.7)	73 (38.8)	29 (40.9)	29 (38.2)	17 (44.7)	44 (39.3)
GSTT1 null	39 (35.8)	71 (37.8)	27 (38.0)	29 (38.2)	12 (31.6)	42 (37.5)
EPHX exon 3[e]						
113YY	68 (61.8)	128 (66.3)	43 (61.4)	47 (59.5)	25 (62.5)	81 (71.1)
113YH	28 (25.5)	50 (25.9)	16 (22.9)	24 (30.4)	12 (30.0)	26 (22.8)
113HH	14 (12.7)	15 (7.8)	11 (15.7)	8 (10.1)	3 (7.5)	7 (6.1)
EPHX exon 4[e]						
139HH	63 (57.3)	102 (55.4)	40 (57.1)	37 (52.1)	23 (57.5)	65 (57.5)
139HR	44 (40.0)	69 (37.5)	28 (40.0)	28 (39.4)	16 (40.0)	41 (36.3)
139RR	3 (2.7)	13 (7.1)	2 (2.9)	6 (8.5)	1 (2.5)	7 (4.6)
EPHX exon 3 and 4 combined[e]						
113HH and *139HH*	12 (10.9)	7 (3.8)[a]	11 (15.7)	3 (4.2)[a]	1 (2.5)	4 (3.5)

[a] Significantly different from cases ($P < 0.05$).
[b] NA, not applicable.
[c] Average frequency calculated as: [3 × freq "in season" + 9 × freq "off-season"] × 1/12; average consumption calculated as: [3 × freq "in season" + 9 × freq "off-season"] × portion size × 1/12.
[d] Information on hepatitis B and hepatitis C virus infections was available for 109 cases and 193 controls; among West Sudanese participants, 1 case and 1 control had evidence of both hepatitis B and C virus infection.
[e] Information on *GSTM1* and *GSTT1* genetic polymorphism was available for 109 cases and 188 controls, on *EPHX* exon 3 for 110 cases and 193 controls, and on *EPHX* exon 4 for 110 cases and 184 controls.

ties. Thus, blood was collected from 113 cases and 199 controls.

Laboratory Analysis. DNA was isolated from 200-μl whole blood using the QIAamp blood kit (Qiagen, Inc., Chatsworth, CA), stored at 4°C, and directly used as a template in PCR analyses. Because of small amounts and low quality of some blood samples, DNA isolation failed for samples of 1 case and 5 controls. In total, DNA of 112 cases and 194 controls was available for genotyping.

A multiplex PCR was done to determine presence or absence of the *GSTM1* and *GSTT1* genes simultaneously according to Arand *et al.* (20). As a positive PCR control, however, we used primers derived from β-globin (21) instead of histidine. Primers derived from *GSTM1*, *GSTT1*, and β-globin were 5′-GAACTCCCTGAAAAGCTAAAGC-3′ and 5′-GTTGGGCTCAAATATACGGTGG-3′, 5′-TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCACCGGATCATGGCCAGCA-3′, and 5′-CAACTTCATCCACGTTCACC-3′ and 5′-GAAGAGCCAAGGACAGGTAC-3′, respectively. After DNA electrophoresis on an ethidium bromide-stained agarose gel, the amplified products were visualized under UV light. The *GSTM1* fragment was 215 bp, the *GSTT1* fragment was 480 bp, and the β-globin fragment was 350 bp in size. *GSTM1*/*T1* genotyping was conducted in duplicate. For two subjects, not enough DNA was left to repeat genotyping. Samples with inconsistent genotyping results ($n = 2$) were genotyped a third time, and these consistent results are presented in this paper.

Genetic polymorphism in *EPHX* exon 3 (*113Y* and *113H* alleles) and exon 4 (*139H* and *139R* alleles) were determined by RFLP analysis after amplification of the exons. For amplification of exon 3, we used primers described by Smith and Harrison (Ref. 22; 5′-GATCGATAAGTTCCGTTTCACC-3′ and 5′-ATCTTAGTCTTGAAGTGAGGAT-3′). A mismatch in the reverse primer incorporated an *Eco*RV restriction site in the amplicon of the *113Y* allele resulting in digestion products of 23 and 140 bp. The *113H* allele remained undigested. Amplification of exon 4 was done using primers described by Hassett *et al.* (Ref. 15; 5′-GGGGTACCAGAGCCTGACCGT-3′ and 5′-AACACCGGGCCCACCCTTGGC-3′), followed by restriction analysis with *Rsa*I. The *139H* allele was digested into two fragments (295 and 62 bp), and the *139R* allele was digested into fragments of 174, 121, and 62 bp in size. The amplified products were visualized under UV light after DNA electrophoresis on an ethidium bromide-stained agarose gel.

Determination of chronic hepatitis B and C virus infection was done using Hepanostika HBV surface antigen and ORTHO HCV 3.0 ELISA test systems with enhanced SAVe test kits, according to the instructions of the manufacturer.

Data Analysis. Analyses were restricted to subjects who completed the questionnaire and of whom genotyping results were

Table 2 Genotypes of *GSTM1*, *GSTT1*, and *EPHX* and risk of HCC

	OR (95% CI)		
	Univariate	Adjusted for age	Multivariate[a]
GSTM1			
Present	1.00 (Referent)	1.00 (Referent)	1.00 (Referent)
Null	1.18 (0.73–1.90)	1.26 (0.73–2.16)	1.26 (0.73–2.19)
GSTT1			
Present	1.00 (Referent)	1.00 (Referent)	1.00 (Referent)
Null	0.92 (0.56–1.50)	0.94 (0.54–1.64)	0.94 (0.54–1.65)
EPHX exon 3			
113YY	1.00 (Referent)	1.00 (Referent)	1.00 (Referent)
113YH	1.05 (0.61–1.82)	0.94 (0.51–1.73)	0.93 (0.50–1.71)
113HH	1.76 (0.80–3.85)	1.68 (0.69–4.09)	1.51 (0.61–3.75)
EPHX exon 4			
139RR	1.00 (Referent)	1.00 (Referent)	1.00 (Referent)
139HR	2.75 (0.75–10.25)	3.67 (0.89–15.06)	3.63 (0.88–15.02)
139HH	2.68 (0.73–9.76)	3.19 (0.80–12.79)	3.22 (0.80–13.01)
EPHX exon 3 and 4 combined			
All other combinations	1.00 (Referent)	1.00 (Referent)	1.00 (Referent)
113HH and *139HH*	3.10 (1.18–8.12)	2.97 (0.99–8.85)	2.56 (0.83–7.95)

[a] Multivariate model adjusted for age (tertiles) and region of origin.

available for at least one polymorphism, *i.e.*, 112 cases and 194 controls. For 108 cases and 188 controls, information on all genotypes was available. For *GSTM1* and *GSTT1*, results were available for 109 cases and 188 controls; *EPHX* genotype at exons 3 and 4 could be determined for 110 cases and 193 controls and for 110 cases and 184 controls, respectively. The *GSTM1*- and *GSTT1*-null genotypes were *a priori* considered as the high-risk genotypes. For *EPHX*, we first conducted the analyses separately for both polymorphic sites. Thus, when analyzing the exon 3 polymorphism, we did not consider the exon 4 polymorphism and *vice versa*. On the basis of the available literature on the association between *EPHX* genotype and phenotype, we considered *113HH* and *139HH* genotypes and the combination of these to be the *EPHX* high-risk genotypes (23). On the basis of the median peanut butter consumption in controls, we classified all subjects as low or high peanut butter consumers. Analyses were done using the SAS statistical software package (release 6.12). After univariate analyses, we adjusted for age (in tertiles, according to age distribution in the control group). Multiple variables (*e.g.*, education level, job type, region of origin and hepatitis infection) were considered to be included in the model if numbers in the various cells were sufficiently large (*i.e.*, >5 after one-way stratification). Variables remained in the model if their inclusion caused a change of $\geq 10\%$ in the β-estimates. Although adjustment for age did not change the β-estimates, this variable was forced into the model because cases were significantly older than controls, and age is known to be related both to several HCC risk factors (*e.g.*, alcohol consumption, smoking) and to the disease itself.

Results

Table 1 shows the characteristics of the study population. Cases were older than controls, less educated, and more often resided in West Sudan. They also consumed significantly more peanut butter, both in amount as in frequency per month. Besides, more cases were chronic carriers of the hepatitis B or C virus. Results on *GSTM1* have been published previously but are now presented jointly with other polymorphisms. Frequencies of the *GSTM1*- and *GSTT1*-null genotypes were similar for cases and controls. There were no statistically significant differences in frequency of *EPHX* variants in exons 3 and 4. However, cases more often had the *EPHX 113HH* genotype in combination with the *EPHX 139HH* genotype than controls (Table 1). Results of the West Sudanese population were similar to results of the total population. However, in contrast to the West Sudanese population, Central Sudanese cases and controls did not differ with respect to peanut butter consumption (both the amount and frequency) and *EPHX* genotype (Table 1). Allelic variants of the studied polymorphic genes occurred at similar frequencies among controls from West and Central Sudan; the biggest difference between the regions was observed for *EPHX* exon 3: *113HH* and *113YH* genotypes occurred in 10 and 30% of Western controls and in 6 and 23% of Central Sudanese controls, respectively. The results presented in Table 1 did not change significantly after adjustment for age.

In Table 2, ORs of HCC are shown for genetic polymorphism of *GSTM1*, *GSTT1*, and *EPHX*. Univariate ORs indicate that only the combination of the *EPHX 113HH* and *139HH* increased the risk of HCC. Adjustment for age and region in a multivariate model did not change the results on *GST* polymorphisms, although the association between the *EPHX 113HH* and *139HH* genotypes and HCC lost statistical significance. Although inclusion of a variable for hepatitis infection changed ORs significantly (by increasing ORs and 95% CIs for the *GSTM1*-null and *EPHX 139HH* and *139HR* genotypes to statistically significant values), it was not included in the multivariate model because this substantially inflated the SEs of the β-estimates. This was probably attributable to limited data: only 16 controls showed evidence of hepatitis infection, of whom 1 presented the *EPHX 113HH* and *139HH* genotype, and 5 carried the *GSTM1*-null genotype.

To investigate if genetic polymorphism of *GSTT1* or *EPHX* modified the association between peanut butter consumption and HCC, we stratified for peanut butter consumption. Results are shown in Table 3. We showed previously that peanut butter consumption was a risk factor for HCC, especially in *GSTM1*-null genotype carriers (3). Interestingly, effect modification by *GSTT1* or *EPHX* genotype was not observed (ORs for the interaction terms were 1.3 and 1.0, respectively). In contrast to *GSTM1*, *GSTT1* and *EPHX* polymorphisms did not affect the association between peanut butter consumption and HCC in subgroups with relatively high peanut butter con-

Table 3 Genotypes of *GSTM1*, *GSTT1*, and *EPHX* as risk factors for HCC, stratified for peanut butter consumption[a]

Genotype	ORs and 95% CIs, age adjusted[b]				ORs and 95% CIs, multivariate[c]	
	Low[a]		High[a]		Low[a]	High[a]
	No. of cases/controls	OR (95% CI)	No. of cases/controls	OR (95% CI)	OR (95% CI)	OR (95% CI)
All genotypes	33/98	1.00 (Referent)	78/96	2.62 (1.50–4.57)	1.00 (Referent)	2.50 (1.43–4.38)
GSTM1						
Present	25/55	1.00 (Referent)	37/60	1.57 (0.77–3.17)	1.00 (Referent)	1.43 (0.70–2.93)
Null	8/38	0.55 (0.21–1.47)	39/35	2.75 (1.30–5.78)	0.55 (0.21–1.47)	2.60 (1.22–5.54)
GSTT1						
Present	18/58	1.00 (Referent)	51/59	2.71 (1.30–5.63)	1.00 (Referent)	2.71 (1.30–5.63)
Null	14/35	1.25 (0.51–3.11)	25/36	2.28 (0.99–5.23)	1.26 (0.51–3.11)	2.28 (0.99–5.23)
EPHX exon 3						
113YY	22/65	1.00 (Referent)	45/63	2.47 (1.24–4.94)	1.00 (Referent)	2.26 (1.12–4.56)
113YH	6/24	0.74 (0.25–2.23)	22/26	2.34 (1.02–5.38)	0.66 (0.22–2.01)	2.28 (0.99–5.25)
113HH	4/8	1.47 (0.35–6.20)	10/7	4.54 (1.33–15.49)	1.35 (0.31–5.78)	3.75 (1.07–13.12)
EPHX exon 4						
139RR	21/54	1.00 (Referent)	41/48	1.90 (0.12–31.48)	1.00 (Referent)	1.70 (0.10–28.24)
139HR	10/30	2.48 (0.24–25.30)	34/39	7.85 (0.83–74.67)	2.37 (0.23–24.07)	7.18 (0.76–67.98)
139HH	1/9	2.88 (0.30–27.44)	2/4	6.54 (0.69–61.37)	2.82 (0.30–26.80)	6.02 (0.64–56.22)
EPHX exons 3 and 4 combined						
All other combinations	29/89	1.00 (Referent)	68/88	2.57 (1.43–4.62)	1.00 (Referent)	2.43 (1.34–4.39)
113HH and *139HH*	3/4	3.02 (0.51–18.01)	9/3	7.68 (1.65–35.69)	2.60 (0.41–16.69)	6.31 (1.32–30.05)

[a] Low consumption defined as consumption of ≤135 g of peanut butter/month, high consumption defined as consumption of >135 g of peanut butter/month, based on the median consumption among controls.
[b] Adjusted for age (tertiles).
[c] Adjusted for age (tertiles) and region of origin.

sumption. These results did not change significantly after adjustment for age and region.

In *GSTM1*-null genotype carriers, presence of the *GSTT1*-null genotype or the *EPHX 113HH* and *139HH* genotypes did not increase the risk of HCC; ORs and 95% CIs were 0.7, 0.3–1.9 and 2.0, and 0.4–11.2, respectively. Strikingly, only those with *GSTM1* non-null genotypes in combination with the *EPHX 113HH* and *139HH* genotypes were at an increased risk of HCC (OR, 5.7; 95% CI, 1.2–28.2). Similarly, subjects with *GSTT1* non-null genotypes and the *EPHX 113HH* and *139HH* genotypes had an increased risk of HCC (OR, 22.2; 95% CI, 2.4–205.8). Because none of the cases and only 3 controls carried a combination of all three high-risk genotypes, *i.e.*, *GSTM1* null, *GSTT1* null, and *EPHX 113HH* and *139HH*, we could not evaluate if the presence of this combination specifically increased the risk of HCC.

Discussion

GSTT1-null genotype alone or in combination with peanut butter consumption was not a risk factor for HCC in this Sudanese case-control study. *EPHX* polymorphism might play a modest role in HCC. However, no interaction with peanut butter consumption was observed, indicating that the mEH enzyme may not be significant in aflatoxin detoxification.

As in all case-control studies, several types of bias might have occurred in this study. Selection bias probably did not occur, because almost all suspected HCC cases were referred to one of the participating hospitals. Therefore, we estimate that only few cases from the two selected regions were missed. Only cases being able to travel to Khartoum were included in this study, and of these, we analyzed results of those providing blood and for whom genotyping results were available. These might be the relatively mildly diseased ones, because some cases might have died before reaching the hospital and blood sampling failed for severely ill cases. It is unlikely that this introduced selection bias, because HCC is fatal in almost all cases, and survival time is very short (1). Moreover, we do not expect *GST* and *EPHX* genotypes to influence HCC survival. We think that the control population correctly reflects the Sudanese population in the two regions with respect to habitual peanut butter consumption and exposure to other HCC risk factors, because both regions are inhabited by populations with culturally determined, relatively constant food habits.

Controls were frequency matched to cases for sex. No age matching was done, because information on age was not available from sugar shop registries. Because cases were older than controls, we adjusted for age in all analyses, although this did not change the results significantly. Controls were enrolled from the same two regions as the cases, proportional to the population size of each region. These regions were chosen to investigate if differences in HCC prevalence may be explained by differences in aflatoxin exposure. Because Central Sudan has more inhabitants than West Sudan, whereas HCC is more prevalent in West Sudan, controls were more likely to be from Central Sudan, and cases were more likely to be West Sudanese. Homogeneity with respect to ethnicity should be considered because like many populations, both the Western and Central study populations may be composed of subjects from different ethnic groups. For cultural reasons, we chose not to record the ethnicity of the participants. Although region (*i.e.*, Central and West) is not a definite predictor of ethnicity, the Central Sudanese population is predominantly Arab whereas people of West Sudan are more related to Chadians. We controlled for possible differences in ethnic composition between the two regions by the inclusion of region as a cofactor in our multivariate model. Stratification for region showed that results of the West Sudanese population were similar to results of the total population. Thus, in West Sudan, peanut butter consumption and *EPHX 113HH*/*139HH* genotypes were risk factors for HCC. In Central Sudan, however, those factors were not related to HCC. Because aflatoxin contamination of peanuts is lower in Central than in West Sudan (19), aflatoxin is probably a less

significant determinant of HCC in Central Sudan. The finding for *EPHX* genotype may be attributable to differences in the ethnic composition of the two regions, as we explained above.

Information bias is not a major consideration in this study, because the population has no knowledge on aflatoxin contamination and its potential hazards. Moreover, we used a standardized questionnaire, and all interviews were conducted by the same person.

Because aflatoxin exposure could not be measured directly, consumption of peanut butter was used to estimate the exposure of the population. Peanut butter is considered to be the main source of aflatoxins in this population: (*a*) it is a frequently consumed staple food highly contaminated with aflatoxins, especially with AFB1 (2, 19, 24); (*b*) other staple foods that may be contaminated with aflatoxins (*i.e.*, corn, sorghum, and millet) were reported to contain aflatoxins at concentrations below the reported hazard level (25); and (*c*) peanut butter consumption was indeed positively associated with the increased risk of HCC in our study, whereas the consumption of corn, sorghum, and millet was not.

In Sudan, life-style patterns are relatively simple because of limited food choice, limited amount and types of jobs, and religious uniformity. We therefore think our data are quite complete with respect to assessment of potential confounders, such as socioeconomic status (estimated by marital status, job type, income, and education level) and potential HCC risk factors (*i.e.*, consumption of other main food types and alcohol consumption, smoking, and hepatitis). All variables assessing socioeconomic status were associated with HCC but did not confound the association between peanut butter consumption and HCC. Nevertheless, residual confounding could still have occurred. Because adjustment of the studied associations for potential confounders weakened our estimates only marginally, we expect that residual confounding did not distort the studied associations significantly.

The frequency of the *GSTM1*-null genotype in our control group was in-between frequencies reported for Europeans and sub-Saharan Africans (13, 16, 26), and the frequency of the *GSTT1*-null genotype was similar to prevalences found in other African studies (13, 14). Because *EPHX* polymorphism is known to vary greatly across people of different ethnicity (14, 16), our results on *EPHX* genotype frequencies cannot be easily extrapolated to other populations. Adjustment of our results for region neutralized the slight difference in *EPHX* genotype frequencies between the two regions.

Like *GSTM1*, the *GSTT1*-null genotype itself was not associated with HCC in this study. In contrast to *GSTM1*, *GSTT1* did not modify the relation between peanut butter consumption and HCC. This may suggest that *GSTT1* polymorphism is not important in aflatoxin metabolism or in HCC etiology. Possibly, conjugation of glutathione to AFB1–8,9-*exo*-epoxide is efficiently catalyzed by GST-μ, and in the case of GST-μ deficiency, GST-θ does not take over the function of GST-μ, but the metabolite is detoxified via hydrolysis instead. Our results correspond with those of a study in Gambia including 357 healthy subjects, showing that *GSTT1* polymorphism was not related to aflatoxin-albumin adduct levels (14). Chen *et al.* (10), however, reported that HBV-positive, *GSTT1*-null genotype carriers had increased albumin adduct levels and were at an increased risk of HCC in a small study of 32 cases and 73 controls.

We found that the combination of *EPHX 113HH* and *139HH* genotypes increased the risk of HCC. In a study in China including 52 cases and 116 controls, McGlynn *et al.* (9) found that carriers of the *113HH* genotype were at an increased risk of HCC. Wild *et al.* (14), however, reported that this genotype was not associated with aflatoxin-albumin adducts. Both research groups did not study the *EPHX* exon 4 polymorphism. We found no indications for the *EPHX* polymorphism to modify the relationship between peanut butter consumption and HCC. Biochemical studies showed that mEH-catalyzed hydrolysis is probably not a rate-limiting step in aflatoxin detoxification, because hydrolysis of the AFB1–8,9-*exo*-epoxide can occur spontaneously at a rate comparable with that of mEH-catalyzed hydrolysis (27). The role of mEH in detoxification of polycyclic aromatic hydrocarbons might be more significant (28). This could explain the increased risk of liver (9), ovarian (29), and colon cancer (30) associated with allelic variants of *EPHX*. However, *in vitro* studies did not reveal a clear genotype-phenotype correlation for the polymorphic sites of *EPHX* (15, 18, 31, 32). This might imply that the studied polymorphic sites are linked to other genetically polymorphic sites, *e.g.*, sites in noncoding regions of the gene, thereby modifying regulation of gene transcription (33).

Wild *et al.* (14) found that the presence of *GSTM1*- and *GSTT1*-null genotypes in combination with the *EPHX 113HH* genotype was related to elevated aflatoxin-albumin adduct levels, although not with statistical significance. We could not evaluate the effect of such a combination, because none of the cases and only 3 controls in our study had this combination of genotypes. However, the combinations of *GSTM1* or *GSTT1* non-null genotypes with the *EPHX 113HH* and *139HH* genotypes increased the risk of HCC, which is not in line with the proposed metabolism (see Fig. 1). These unexpected findings might be attributable to chance because of the small number of observations per category, but they might also indicate that mEH is involved in other metabolic pathways rather than AFB1 detoxification.

We have estimated that as much as/up to 50% of HCC cases in Sudan might be attributed to HBV infections (34). Hepatitis infection has been reported to modify the relation between genetic polymorphism and HCC (9, 35). However, we could not study such effect modification in our study population because the number of controls with evidence of hepatitis infection was too small.

Although being the largest study on aflatoxin-associated HCC and genetic polymorphism to date, the population was still small to study relatively weak gene-environment interactions. Especially our results on *EPHX* should be interpreted with care, because 113 HH and 139 RR genotypes occurred at low frequency, and after stratification, some cells had <5 observations.

In conclusion, our results do not indicate that *GSTT1* plays a role in HCC. *EPHX* polymorphism might be related to HCC, although the encoded enzyme may not be important in AFB1 detoxification in which GST-μ plays a major role. This seems to be in line with *in vitro* studies (11). Because *EPHX* polymorphism appears to be associated with cancer risk, the specific effect of this polymorphism on gene expression and enzyme function and the role of the mEH enzyme in various metabolic pathways need to be addressed in future studies.

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