TP53 R249S Mutations, Exposure to Aflatoxin, and Occurrence of Hepatocellular Carcinoma in a Cohort of Chronic Hepatitis B Virus Carriers from Qidong, China

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Abstract

Hepatocellular carcinoma (HCC) has a high mortality in East Asia and Sub-Saharan Africa, two regions where the main etiologic factors are chronic infections with hepatitis B virus and dietary exposure to aflatoxin. A single base substitution at the third nucleotide of codon 249 of *TP53* (*R249S*) is common in HCC in these regions and has been associated with aflatoxin-DNA adducts. To determine whether *R249S* may be detected in plasma DNA before HCC diagnosis, we conducted a case-control study nested in a cohort of adult chronic hepatitis B virus carriers from Qidong County, People's Republic of China. Of the 234 plasma specimens that yielded adequate DNA, only 2 (0.9%) were positive for *R249S* by restriction fragment length polymorphisms, and both of them were controls. Of the 249 subjects tested for aflatoxin-albumin adducts, 168 (67%) were positive, with equal distribution between cases and controls. Aflatoxin-albumin adduct levels were low in the study, suggesting an overall low ongoing exposure to aflatoxin in this cohort. The *R249S* mutation was detected in 11 of 18 (61%) available tumor tissues. To assess whether low levels of mutant DNA were detectable in pre-diagnosis plasma, 14 plasma specimens from these patients were analyzed by short oligonucleotide mass analysis. Nine of them (64%) were found to be positive. Overall, these results suggest that HCC containing *R249S* can occur in the absence of significant recent exposure to aflatoxins. The use of short oligonucleotide mass analysis in the context of low ongoing aflatoxin exposure may allow the detection of *R249S* in plasma several months ahead of clinical diagnosis. (Cancer Epidemiol Biomarkers Prev 2009;18(5):1638–43)

Introduction

Hepatocellular carcinoma (HCC) is a major public health problem in many parts of the world, with high incidence areas in East Asia and Sub-Saharan Africa, where the main etiologic factors are chronic infection with hepatitis viruses [mostly hepatitis B virus (HBV)] and dietary exposure to aflatoxins, including aflatoxin B_1. Almost 54% of all liver cancer cases occur in the People's Republic of China (PRC; ref. 1). In Qidong County, PRC, about 16% of the adult population are seropositive for HBsAg (2). A study on 181 consecutive HCC cases from Qidong showed markers of HBV infection in all of them, whereas only 6 of 119 HCC cases were coinfected with hepatitis C virus (HCV). Dietary exposure to aflatoxins was ubiquitous (3). A missense mutation at codon 249 in *TP53*, AGG to AGT, leading to a substitution of an arginine for a serine (*R249S*), is extremely common in HCC in areas with high prevalence of HBV chronic carriage and aflatoxin exposure, but not in areas with high HBV prevalence alone (4, 5). This mutation is considered to be a consequence of aflatoxin N^7-guanine adducts formed at the third base of codon 249 of the *TP53* gene (6). However, the reasons why this particular mutation is selected in HCC are not known. There is evidence of a synergistic effect between HBV carriage and aflatoxin exposure in inducing this mutation in HCC (4). However, despite ecological correlations (7), there is limited evidence for an association between recent aflatoxin exposure and the mutation at the individual level (8).

Recent studies have shown that *R249S* is detectable in free DNA extracted from serum or plasma of HCC patients. Using a sensitive method based on mass spectrometry, Jackson et al. (9) detected *R249S* at least 1 year before diagnosis in the sera of four of eight Qidong HCC patients who were positive for this mutation at the time of diagnosis. In a case-control study in The Gambia, another area of high incidence of HCC and common exposure to HBV and aflatoxin B_1, Kirk et al. (10) found that *R249S* was sometimes detectable in subjects with liver cirrhosis (15.3%) or in control subjects (3.5%), although with a lower prevalence than in HCC patients (39.8%). In our study, we have assessed the prevalence of *R249S* mutations in plasma and tumor DNA, HCV infection, and exposure to aflatoxin [based on quantitation of aflatoxin-albumin (Af-alb) adducts in the serum]

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in a case-control study nested within a cohort of HBV chronic carriers in Qidong, PRC, undergoing follow-up for the occurrence of HCC and other cancers.

Materials and Methods

Study Design and Subjects. In 1989, a program was launched to screen for liver cancer among men aged 30 to 59 resident in Qidong County, Jiangsu Province, PRC. Within this program, 36,000 subjects were tested for carriage of HBsAg, and 3,712 subjects were followed up by testing for increased serum α-fetoprotein at 6-month intervals for up to 6 years, with a record of the occurrence of liver cancers (11). Plasma specimens (~0.5-1.0 ml) taken at the time of the initial recruitment and at subsequent screenings were stored at −20°C. A total of 130 primary liver cancer patients were identified between 1993 and 1998. These cases were identified among the 3,712 individuals either via the screening process or by follow-up of the cohort through the Qidong Cancer Registry (11). Most patients were diagnosed by a combination of α-fetoprotein measurement, alanine aminotransferase tests, and ultrasound. For 20 subjects, tumor biopsies (12 cases) or surgical specimens (8 cases) were available, the latter with both tumoral and peritumoral tissue specimens. For each case, one age-matched control was randomly selected from within the same study cohort. To be eligible as a control, a subject had to be in the same age category as the case, be still alive and free of cancer at the date of cancer diagnosis for the corresponding case, and have the same number of collected specimens as the case in the period before the case was diagnosed. Considering the rarity of HCV infection in the Chinese population, two more controls for each case were selected to explore the possible synergism of HCV and HBV in the development of HCC. The presence of HCV antibody was detected using a second-generation ELISA assay.

***TP53* Analysis.** The tissue specimens were histologically evaluated, classified according to the Edmonson-Steiner criteria (ES grades), and immunostained for p53 using a polyclonal antibody CM1 (NovoCastra). Immunostaining, DNA extraction from tissue and plasma specimens, and *TP53* analyses by restriction fragment length polymorphism (RFLP) and sequencing were done as described elsewhere (12). Briefly, exon 7 of the *TP53* gene was amplified and the PCR products were digested with a restriction endonuclease, *Hae*III, recognizing sequence CCGG, encompassing the 249 codon. Digestion of the wild-type exon 7 generates two bands (92 and 66 bp), whereas mutant material, in which the restriction site has been destroyed by the mutation, yields one band of 158 bp. These mutant fragments were cut out of the gel, reamplified, and sequenced by automated dideoxy sequencing to confirm the presence of the mutation. The samples were classified as mutant if the same result was found for two different PCR products. Mutations in plasma specimens were confirmed by short oligonucleotide mass analysis (SOMA) as described elsewhere (9, 13). Tissue specimens appearing wild type at digestion were directly sequenced (from a third PCR product) to screen for possible other mutations in exon 7. To search for mutations in other exons of the *TP53* gene, additional analyses were done using temporal temperature gradient electrophoresis as described elsewhere (14, 15). Additionally, 15 plasma specimens corresponding to the analyzed tumors were analyzed for the presence of the *R249S* DNA by SOMA (9, 13).

Aflatoxin-albumin Adducts. The concentration of Aflatoxin-albumin adducts in plasma was measured as previously described (16). Albumin (2 mg) was digested overnight using Pronase, and aflatoxin-containing residues were isolated by solid-phase extraction using a Sep-Pak cartridge. Samples were analyzed in quadruplicate by ELISA on two occasions on separate days using aflatoxin B_1-lysine in the standard curve. The limit of detection was 3 pg aflatoxin B_1-lysine equivalent/mg of albumin.

Statistical Analysis. Logistic regression models (including exact age) were used to estimate the odds ratios and 95% confidence intervals associated with exposure to HCV and Af-alb adducts and the development of liver cancer.

Results

Detection of *R249S* DNA in Tumor Tissues. To determine the prevalence of *R249S TP53* mutation in HCC cases appearing among chronic HBV carriers, 20 available tumor specimens were analyzed. Ten tumors (50%: 6 of 12 biopsies and 4 of 8 surgical specimens) stained positive for p53 (at least 10% of stained tumor cells) with antibody CM1. Only two tumors (10%) showed positivity in >50% of tumor cells and both were diagnosed as HCC ES 2 (Table 1). DNA of sufficient quality for PCR amplification was obtained from 18 specimens. Eleven of these 18 tumors (61%) contained G-to-T transversions at the third nucleotide of codon 249 (the *R249S* mutation; Table 1). The distribution of mutations was uneven between the two series of specimens: Whereas only 5 of 11 biopsies (ID nos. 1-12 in Table 1) contained the *R249S* DNA, the mutation was found in 6 of 7 surgical specimens (the only one without the mutation contained a majority of nontumoral cells, so that the wild-type allele may have masked the mutant allele). Peritumoral liver tissues were available for eight cases, including five cirrhotic and three fibrotic tissues. *R249S* mutations were detected in three of the five cirrhotic tissues but not in fibrotic tissues. In addition to *R249S*, two other mutations were found by sequencing of exon 7, both in *R249S*-negative tumors (CCC→TCC, Pro→Ser, at codon 250 in patient no. 1, and GTC→TTC at donor splice site in patient no. 11; Table 1). The presence of *TP53* mutations did not correlate with histologic features of the tumors or with p53 immunohistochemistry.

Detection of *R249S* in Plasma DNA. Cell-free DNA was extracted from plasma collected during the follow-up of the cohort of chronic carriers. A total of 130 subjects who developed HCC during the follow-up (''cases'') were matched with subjects who did not develop HCC (''controls''). For both cases and controls, the most recently collected plasma samples were analyzed for the presence of *R249S* DNA by RFLP. In cases, the time elapsed between plasma collection and cancer diagnosis ranged from 0 to 74 months (two plasma samples were obtained at the date of cancer diagnosis). Only three specimens were found to be positive by RFLP, and they

Table 1. Histologically confirmed HCCs arising within the Qidong cohort of hepatitis B chronic carriers: patients' characteristics, tumor grade, HCV, and *TP53* status

ID	Sex	Age (y)	Diagnosis	Anti-HCV	p53 immunostaining (%)	TP53 mutations, exon 7
6	M	33	HCC, clear cell	—	—	AGG→AGT at 249
3	M	47	HCC, ES 2	ND	>50+	AGG→AGT at 249
8	M	36	HCC, ES 2	ND	—	Wt
9	M	38	HCC, ES 2	—	>50+	Wt
10	M	47	HCC, ES 2	ND	0-10+	AGG→AGT at 249
4	M	36	HCC, ES 2	ND	20-50+	NA
12	M	42	HCC, ES 2	—	—	AGG→AGT at 249
15CA	M	49	HCC, ES 2	—	0-10+	AGG→AGT at 249
15AF			Cirrhosis		—	Wt
16CA	M	61	HCC, ES 2	ND	—	AGG→AGT at 249
16AF			Fibrosis		—	Wt
13CA	M	39	HCC, ES 2	ND	0-10+	AGG→AGT at 249
13AF			Cirrhosis, inactive		—	AGG→AGT at 249
14CA	M	35	HCC, ES 2	—	20-50+	AGG→AGT at 249
14AF			Cirrhosis, moderately active		—	AGG→AGT at 249
19CA	M	59	HCC, ES 2	—	20-50+	AGG→AGT at 249
19AF			Cirrhosis, vascular neoplastic emboli		20-50+	AGG→AGT at 249
18CA	M	59	HCC ES 2	—	—	NA
18AF			Cirrhosis, active		—	Wt
1	M	41	HCC, ES 3	—	20-50+	CCC→TCC at 250
5	M	50	HCC, ES 3	—	—	Wt
7	M	38	HCC, ES 3	—	—	Wt
11	M	50	HCC, ES 3	—	—	GTC→TTC at donor splice site
17CA	M	46	HCC, ES 3	—	20-50+	AGG→AGT at 249
17AF			Fibrosis		—	Wt
2	M	37	HCC	—	20-50+	AGG→AGT at 249
20CA	M	62	HCC (vascular embolus, small tumor area)	—	20-50+	Wt
20AF			Fibrosis		—	Wt

NOTE: Samples 1 to 12 are biopsies and samples 13 to 20 are surgical specimens. In the series of surgical specimens, peritumoral liver specimens were also available (marked AF in the ID number as opposed to CA for tumor specimens).

were reanalyzed by SOMA to confirm the presence of the mutation, with two (0.9%) being confirmed as *R249S*-positive by RFLP/sequencing and SOMA. Both were controls, neither of whom developed cancer during the follow-up period, which was 6 years for one subject but only 1 year for the other. Plasma specimens were available for 14 of the 18 patients from whom tumors were analyzed for the presence of *R249S*. Only two of these matched plasma specimens were collected at the time of cancer diagnosis. Twelve of them were analyzed by RFLP and none of them was found positive for *R249S* DNA. Next, all 14 plasma specimens were analyzed by SOMA. Nine of 14 samples (64%) were found to contain *R249S* DNA at levels between 157 and 3,546 gene copies/ml of plasma. This result shows that low levels of *R249S* were detectable in the plasma ahead of cancer diagnosis. Due to a limited amount of plasma and logistical constraints, analysis by SOMA could not be extended to all specimens.

The concordance between positivity in the plasma as detected by SOMA and positivity in the tumor as detected by RFLP and sequencing was poor. Three of the SOMA-positive plasma specimens were from subjects who later developed *R249S*-negative tumors. In contrast, two of the plasma specimens that were negative by SOMA corresponded to tumors that were found to be positive for *R249S* by RFLP. Thus, the presence of *R249S* in the plasma was not systematically predictive of the detection of the same mutation in the tumor (Table 2).

Detection of HCV. To assess the possible contribution of HCV as a risk of HCC in hepatitis B chronic carriers, we analyzed the prevalence of antibodies against HCV in 127 cases and 380 controls. There were only 10 positive subjects among 507 tested (2%; 2 cases and 8 controls; Table 3A). The odds ratio associated with HCV infection was 0.94 (95% confidence interval: 0.45-1.97). Thus, HCV does not seem to substantially contribute to the risk of HCC in this cohort.

Detection of Aflatoxin Adducts. To determine whether exposure to aflatoxin in the months and years that precede diagnosis was an important risk factor for HCC, the plasma from the last available aliquot was also analyzed for Af-alb adducts. Testing was possible in 123 cases and 126 controls. Of the 249 individuals tested, 168 were positive for this biomarker of exposure to aflatoxin in one or more specimens taken before the diagnosis of the case or the same reference date for the controls (Table 3B). The adduct levels were uniformly low, with most of the positive specimens showing levels between 5 and 10 pg/mg and only six specimens with levels higher than 10 pg/mg. Using a value of 3 pg/mg to dichotomize individuals into exposed and unexposed, there was no difference between cases and controls (odds ratio: 0.90; 95% confidence interval: 0.52-1.56). It is considered that Af-alb adducts reflect recent past exposure (past 2 to 3 months). Our results therefore suggest that exposure to aflatoxin in the months before diagnosis was not a major contributor to the risk of HCC in this cohort. Because the

Table 2. *R249S* in plasma specimens matched with the analyzed tumors

plasma ID	Time lapse between plasma collection and cancer diagnosis (mo)	*R249S* in plasma by SOMA (copies/mL plasma)	Corresponding tumor ID	*R249S* in tumor by RFLP/seq	Other *TP53* mutations (exon 7) in tumor
28-1	0	0	1	Wt	CCC>TCC at 250
162-1	2	0	6	+	
151-1	2	0	9	Wt	
12-4	7	0	15	+	
168-6	21	0	20	Wt	
87-2	4	157	7	Wt	
258-5	54	247	19	+	
170-6	16	490	12	+	
10-6	32	593	17	+	
137-1	1	725	2	+	
90-3	23	817	11	Wt	GT>TT at donor splice site
138-5	0	946	14	+	
40-1	4	2310	5	Wt	
48-1	1	3546	3	+	

region of Qidong is historically known as an area of high exposure to dietary aflatoxin, these results also suggest that exposure to aflatoxin in this area of China has recently decreased as a result of public health intervention and/or changes in dietary and lifestyle patterns.

Discussion

In this study, we have used a nested case-control design to examine the contribution of aflatoxin and HCV to the risk of HCC in a cohort of chronic HBV carriers from Qidong County, PRC, an area of traditionally high exposure to aflatoxins. As a marker of mutagenesis by aflatoxin, we have analyzed the *R249S TP53* mutation, which is common in liver cancer in those parts of the world where exposure to aflatoxin is high. First, we showed that the *R249S* mutation was present in 61% (11 of 18) of HCC cases, confirming the results of previous studies in HCC from the Qidong area. Second, we described the occasional presence of low levels of free plasma DNA containing *R249S* in plasma specimens collected ahead of diagnosis. Detection of such mutant plasma DNA was, however, not a predictor of HCC development in this cohort. Third, the prevalence of HCV infection was low and does not seem to play a significant role in HCC etiology in this population. Fourth, individual exposure to aflatoxin, as measured by the detection of Af-alb adducts in plasma, indicates moderate to low exposure in the years and months before HCC diagnosis.

In a previous study using plasma specimens from West Africa (10), we found a good concordance between *R249S* mutations as detected by RFLP and by SOMA. This was not the case in the present study, perhaps because mutant DNA levels in the plasma were particularly low. In the West African study, about 35% of liver cancer patients were found to carry up to 10,000 copies of mutant plasma DNA/ml of plasma. At these high levels, RFLP gives robust results that are in good agreement with more sensitive methods such as SOMA. At low levels, however, the signals generated by RFLP are below the detection threshold. This explains why some specimens containing trace amounts of *R249S* were found positive by SOMA but not by RFLP. Thus, on the basis of RFLP alone, we cannot consider that plasma was negative for *R249S*, but only that plasma may contain levels of *R249S* that are too low for detection by this method. Unfortunately, due to small initial amounts of plasma and to logistical constraints, it has not been possible to perform SOMA in the plasma of all cases and controls.

Table 3. Prevalence of HCV, *R249S TP53*, and Af-alb adducts in plasma albumin in cases and controls from the Qidong cohort

(A) HCV

	Cases, *n* (%)	Controls, *n* (%)	Total *n* (%)
HCV^+	2 (1.6)	8 (2.1)	10 (2.0)
HCV^-	125 (98.4)	372 (97.9)	497 (98.0)
Total	127 (100.0)	380 (100.0)	507 (100.0)
	Odds ratio, 0.94 (95% confidence interval: 0.45-1.97)		

(B) Af-alb adducts ≥3 pg/mg albumin

	Cases, *n* (%)	Controls, *n* (%)	Total *n* (%)
Af-alb^+	84 (66.7)	84 (68.3)	168 (67.5)
Af-alb^-	42 (33.3)	39 (31.7)	81 (32.5)
Total	126 (100.0)	123 (100.0)	249 (100.0)
	Odds ratio, 0.90 (95% confidence interval: 0.52-1.56)		

Two explanations, not mutually exclusive, have been proposed for the presence of *R249S* in the plasma: it may represent a marker of ongoing mutagenesis by aflatoxins or a marker of early carcinogenesis in the liver. In the first instance, it is expected that levels of *R249S* may increase in subjects with high levels of Af-alb adducts in the plasma. In the second instance, levels of *R249S* in the plasma should be proportional to the tumor mass and the amount of tumor material released into the bloodstream. In the present study, we found that levels of Af-alb adducts were moderate to low in the whole cohort, indicating that contamination by aflatoxins was not widespread anymore in this population.

The low levels of aflatoxin biomarkers we observed may be a result of the national campaign aimed at reducing the aflatoxin contamination of food in China, combined with the increasing affluence in this East Coast province that has resulted in a widespread change from a maize- to rice-based diet. Wang et al. (17) have shown that the dietary patterns in the Jiangsu province changed remarkably in the 1990s, with a gradual decrease in grain consumption and an important increase in consumption of meat. The uniformly low levels of aflatoxin exposure biomarkers also explain why there is no association in this study between aflatoxin exposure and HCC risk among HBV chronic carriers, in contrast to earlier prospective studies (18, 19). The presence of *R249S* in 61% of the tumors despite low levels of Af-alb adducts suggests that this mutation may have been acquired by liver cells well ahead of the recruitment of the subjects in the present cohort, at a time when exposure to aflatoxin was higher. A previous study in the same region has found mutations in 55% (15 of 20) of the cases using the same sensitive SOMA method as described here (20). Other studies, using less sensitive methods, have reported roughly similar prevalences (21-23). In all studies, G→T transversion was the most common if not the only mutation type observed. Interestingly, in our study, the mutation prevalence was higher in surgical specimens than in biopsies. This may be due to heterogeneity within tumors, which may not be captured in a small biopsy, or may reflect a selection bias because the surgical specimens were carefully selected by the surgeon as being representative for the whole tumor.

The absence of significant recent exposure to aflatoxin raises the question of the contribution of this toxin in the time sequence of events leading to HCC. In fact, *TP53* mutations may occur early in life and persist in a subset of liver cells until cancer onset many years later. The fact that we detected the *R249S* mutation is found in peritumoral, cirrhotic liver supports the hypothesis that acquisition of the *R249S* mutation is an event that may take place well ahead of cancer development. This notion is in agreement with results of other studies showing that the mutation was detectable in the plasma of some patients with cirrhosis but not cancer (9, 24, 25). It has been suggested that exposure to aflatoxin in young children may be particularly significant for the permanent acquisition of *R249S* in liver cells. However, a recent study of 149 young children (aged 2-5 years), from a region in West Africa with a higher frequency of serum Af-alb positivity, did not identify *R249S* mutations (26). Further age-stratified analyses of plasma DNA, and when applicable, liver tissues, are required to address the question of timing of occurrence of this mutation in the natural history of HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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