Seasonal Variation in TP53 R249S-Mutated Serum DNA with Aflatoxin Exposure and Hepatitis B Virus Infection

Stéphanie Villar,1* Emilie Le Roux-Goglin,1,6 Doriane A. Gouas,1 Amelie Plymuth,1,6 Gilles Ferro,1 Mathieu Boniol,1,8 Myriam Lereau,1 Ebrima Bah,2 Andrew J. Hall,3 Christopher P. Wild,1 Maimuna Mendy,4 Helene Norder,5 Marianne van der SANdo,5 Hilton Whittle,2 Marlin D. Friesen,7 John D. Groopman,6 and Pierre Hainaut1

1Molecular Carcinogenesis Group, International Agency for Research on Cancer, Lyon, France; 2Gambia Hepatitis Intervention Study, Laboratories Fajara, Banjul, the Gambia; 3Department of Infectious Disease Epidemiology, London School of Hygiene and Tropical Medicine, London, United Kingdom; 4Medical Research Council, Laboratories Fajara, Banjul, the Gambia; 5Swedish Institute for Infectious Disease Control, Solna, Sweden; 6National Institute for Public Health and the Environment, Bilthoven, the Netherlands; 7Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA

BACKGROUND: Chronic hepatitis B virus (HBV) infection and dietary aflatoxin B1 (AFB1) exposure are etiological factors for hepatocellular carcinoma (HCC) in countries with hot, humid climates. HCC often harbors a TP53 (tumor protein p53) mutation at codon 249 (R249S). In chronic carriers, 1762T/1764A mutations in the HBV X gene are associated with increased HCC risk. Both mutations have been detected in circulating cell-free DNA (CFDNA) from asymptomatic HBV carriers.

OBJECTIVE: We evaluated seasonal variation in R249S and HBV in relation to AFB1 exposure.

METHODS: R249S was quantitated by mass spectrometry in CF DNA in a cross-sectional survey of 473 asymptomatic subjects (237 HBV carriers and 236 noncarriers) recruited in three villages in the Gambia over a 10-month period. 1762T/1764A HBV mutations were detected by quantitative polymerase chain reaction. In addition, the HBV X gene was sequenced in 99 subjects positive for HBV surface antigen (HBsAg).

RESULTS: We observed a seasonal variation of serum R249S levels. Positivity for R249S and average concentration were significantly higher in HBsAg-positive subjects surveyed during April–July (61%; 5,690 ± 11,300 R249S copies/mL serum) than in those surveyed October–March (32% and 480 ± 1,030 copies/mL serum [odds ratio = 3.59; 95% confidence interval: 2.05, 6.30; p < 0.001]). Positivity for HBV e antigen (HBsAg) (a marker of HBV replication) and viral DNA load also varied seasonally, with 15–30% of subjects surveyed between April and June HBsAg positive, compared with <10% surveyed during other months. We detected 1762T/1764A mutations in 8% of carriers, half of whom were positive for R249S. We found HBV genotype E in 95 of 99 HBsAg-positive subjects.

CONCLUSION: R249S is detectable in CF DNA of asymptomatic subjects. Evidence of temporal and quantitative variations suggests an interaction among AFB1 exposure, HBV positivity, and replication on TP53 mutation formation or persistence.


About 80% of the 700,000 estimated annual cases of hepatocellular carcinoma (HCC) occur in low-resource, intertropical areas, mainly in sub-Saharan Africa and Southeast Asia (McGlory and London 2005; Sherman 2005). The two major risk factors, chronic hepatitis B virus (HBV) infection and exposure to aflatoxin B1 (AFB1), have been classified as group 1 carcinogens by the International Agency for Research on Cancer (IARC 1994, 2002), with a synergistic effect on HCC risk (Kew 2003). Both factors are preventable in principle (Wild and Hall 2000). The presence of a specific AGG → AGT mutation induced by AFB1 at codon 249 of TP53 tumor suppressor gene (R249S) is observed in >50% of HCC in geographic areas with high incidence. Worldwide, there is a remarkable ecological correlation among AFB1 exposure, incidence of HCC, and prevalence of the TP53 R249S mutation in HCC tumors (Gouas et al. 2009). This mutation occurs early in the pathway leading to HCC and may thus provide an early biomarker of AFB1 exposure and hepatocarcinogenesis (Jackson et al. 2003).

Several studies have shown that plasma or serum of cancer patients contains significant amounts of circulating cell-free DNA (CFDNA), which often carries mutations and epigenetic alterations identical to those detected in tumor tissues (Gormally et al. 2007). Although the mechanisms of release and the stability of mutant DNA in the bloodstream are still unclear, CFDNA may serve as a convenient biomarker in cancer subjects. Importantly, traces of free fragments of DNA are also detectable in most healthy subjects (Gormally et al. 2007). In several instances, this DNA has been found to harbor mutations that predict cancer occurrence (Gormally et al. 2006; Jackson et al. 2003).

Double mutations in the HBV X gene (adenine to thymine, 1762T; and guanine to adenine, 1764A) have been observed in HCC and liver cirrhosis (Arbuthnot and Kew 2001; Baptista et al. 1999; Hou et al. 1999). This mutation is also detectable in CF DNA up to 5 years before HCC diagnosis (Kuang et al. 2004; Yuan et al. 2009). HCC risk is increased in chronic carriers with 1762T/1764A mutations, and it increases with higher serum concentrations of 1762T/1764A (Yuan et al. 2009).

In prospective studies in China, R249S was detected in the plasma of asymptomatic HBV carriers up to several years before HCC diagnosis (Jackson et al. 2003). In a case–control study in the Gambia, West Africa, we used a quantitative method to detect the R249S mutation by electrospray mass spectrometry [short oligonucleotide mass analysis (SOMA)] (Lleonart et al. 2005). Plasma concentrations of R249S were increased in HCC cases (median, 2,800 copies/mL; range, 500–11,000 copies/mL) compared with controls (median, 500 copies/mL; range, 250–2,000 copies/mL). However, low levels of R249S

Address correspondence to P. Hainaut, Mechanisms of Carcinogenesis Section, Molecular Carcinogenesis Group, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon CEDEX 08, France. Telephone: 33 (0) 4 72-73-85-32. Fax: 33 (0) 4 72-73-85-22. E-mail: hainaut@iarc.fr

**These authors contributed equally to this work.

**Current address: Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, Stockholm, Sweden.

*Current address: International Prevention Research Institute, Lyon, France.

Blood samples and data were collected by MRC (Medical Research Council), Fajara, the Gambia. We thank I. Chemin (Institut National de la Santé et de la Recherche Médicale U871) for help with hepatitis B virus molecular analysis. We also thank the following MRC staff: P. Waigh for data management; A. Jing-Berry and A. Bah for laboratory assistance; and P. Rayco-Solon and the field staff at Dunn Nutrition unit, Ceneka.

This work was supported by project CirBioCancer from the French National Cancer Institute (PL06-028), by the U.S. National Institutes of Health (PO1 ES006052), and by the National Institute of Environmental Health Sciences (ES 06052 to C.P.W.). E.G.-R. participated in this work as part of her Ph.D. research, and was supported by Université Claude Bernard Lyon 1.

The authors declare they have no actual or potential competing financial interests.

Received 7 February 2011; accepted 18 July 2011.
 (> 500 copies/mL) were detected in 46% of asymptomatic controls, which suggests that the mutation may also serve as a marker of ongoing exposure to aflatoxin (Lleonart et al. 2005).

In the Gambia, chronic HBV infection is endemic, affecting 11–16% of the adult population (Kirk et al. 2006). AFB1 exposure also is widespread, and the annual incidence of HCC peaks at 80 cases per 100,000 among 35- to 55-year-old males (Bahi et al. 2001). R249S was detectable in the plasma of 40% of HCC patients, with a concordance of 88% between plasma and tumor DNA (Szymańska et al. 2004). R249S was also found in 15% of patients with liver cirrhosis and in 3% of control subjects with no detected liver disease (Kirk et al. 2005). Furthermore, the 1762T/1764A HBV mutation was found in 48% (10 of 21) of asymptomatic carriers, 75.4% (86 of 114) of HCC patients, and 77% (33 of 43) of patients with cirrhosis (Mendy et al. 2008). This mutation has also been found in the serum of 66% of HCC patients and 11% of asymptomatic carriers from South Africa (Baptista et al. 1999). These observations led us to hypothesize that circulating R249S may be a biomarker of early liver disease or, alternatively, a biomarker of ongoing AFB1 exposure, and to further assess the prevalence of 1762T/1764A mutation in asymptomatic subjects from the Gambia who are positive for HBV surface antigen (HBsAg). Therefore, we evaluated seasonal variation in R249S and HBV in relation to AFB1 exposure.

Materials and Methods

Study participants. A total of 473 asymptomatic study participants were recruited from the population of three rural villages in the Gambia (Keneba, Manduar, and Katong-Kunda) where exposure to aflatoxin is widespread and well documented; a previous study in Keneba in the Gambia showed a correlation between measurement of dietary intake of aflatoxin and levels of albumin-bound aflatoxin in serum (Wild et al. 1992). These villages are part of a long-term quadrennial survey program for HBV infection initiated in the early 1980s as a pilot and sentinel program for the Gambian Hepatitis Intervention Study (Whittle et al. 2002). In this rural population, dietary exposure is widespread because of the use of groundnuts as the main basis of diet, whereas exposure to other HCC risk factors such as alcohol is negligible. We used a series of serum specimens (n = 491) collected for the survey from October 2002 to July 2003 (van der Sande et al. 2006) to assemble a case–control study including 237 HBsAg-positive (HBV carriers) and 236 HBsAg-negative (non-carriers) individuals matched by date of sampling, age, and sex. Previous studies have shown that serum or plasma are both suitable sources for CFDNA (reviewed by Gormally et al. 2007). None of the participants had a clinical diagnosis of chronic liver disease at the time of sampling, although no detailed clinical assessment of their liver status was conducted. All participants gave informed consent for inclusion in the quadrennial survey. The present protocol was reviewed and approved by the ethical review boards of the Medical Research Council in the Gambia and of IARC. This study complied with all international standards.

HBV serology. We tested subjects for HBV core antibodies (anti-HBc) and, if positive, HBsAg and HBV e antigens (HBeAg). Anti-HBc was measured using the radioimmunoassay AB-COREK test kit (Sorin Biochemica, Saluggia, Italy) according to the manufacturer’s protocol. Anti-HBc positive samples were tested for HBsAg by reverse passive hemagglutination assay (Wellcotest®; Murex Diagnostics, London, UK) and/or by the Determine™ HBsAg immunochromatographic assay (Abbott Laboratories, Abbott Park, IL, USA). HBsAg-positive subjects were tested for HBeAg using an enzyme immunoassay (Eupar DIAGnostici, Rome, Italy).

CFDNA extraction and quantitation. CFDNA was extracted from 300 µL serum using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the protocol described previously (Szymańska et al. 2004). DNA was eluted in 200 µL elution buffer. Quantitation of extracted CFDNA was performed by fluorimetry using PicoGreen (Interchim, Montluçon, France).

Quantification of R249S in CFDNA. R249S was quantitated by SOMA as described previously (Lleonart et al. 2005), with the following modifications. Before polymerase chain reaction (PCR) amplification of a 93-base-pair segment of exon 7 of TP53 encompassing codon 249, 228 copies of an internal standard plasmid were added to all DNA extracts to provide a reference for quantitation. After restriction digestion with HpaIII, which specifically cuts the wild-type sequence of codon 249, the mutated and internal-standard–enriched PCR products were reamplified, cut with GgoI to produce short 8-mer oligonucleotides, purified, and quantitated by HPLC/electrospray ionization mass spectrometry. Results were expressed as concentrations (copies of R249S per milliliter of serum). Levels ranged from nondetectable to 63,800 copies/mL. The elimination of the wild-type sequence of codon 249 by restriction digestion increased the sensitivity of the method relative to the original method described by Lleonart et al. (2005). Consequently, the limit of detection was 70 copies/mL serum. Samples with serum concentrations > 70 copies/mL were classified as positive for R249S.

HBV DNA quantitation and 1762T/1764A analysis. We performed HBV DNA quantitation as described previously (Mendy et al. 2006), 1762T/1764A mutation analysis was performed by quantitative PCR (Yuan et al. 2009). S gene sequencing. Analysis of the S gene provides genotype information that significantly matches analysis of the entire genome (Hussain et al. 2003; Norder et al. 1993). We developed a new semi-nested PCR amplifying the entire S gene. The first reaction was achieved on 5 µL DNA with primers S_HBVpol1 (5’-ctcgatatggctgctcag3’) and S_HBVporv2 (5’-aaacgcaagaaccagccc3’); round settings were 95°C (15 min); 40 cycles of 95°C (30 sec), 60°C (30 sec), 72°C (1 min); and then 72°C for 7 min. The second step used 1 µL of first reaction product and primers S_HBV123s (5’-tctgaggttggtgccctg3’) and S_HBVporv2; round settings were 95°C (15 min); 45 cycles of 95°C (30 sec), 58°C (30 sec), 72°C (1 min); and then 72°C for 7 min. PCR products (5 µL) were purified using standard ExoSap-IT® enzyme, (USB® Corporation, Cleveland, OH, USA) and nucleotide sequences were determined for both strands by automated dyeoxy sequencing (AblPrism 3100 sequencer; Applied Biosystems, Carlsbad, CA, USA). Direct sequencing on amplified fragments was performed using the primers S_HBV123s, S_HBVporv2, and

Table 1. Descriptive characteristics of participants in the study group by HBsAg status.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HBsAg positive</th>
<th>HBsAg negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>237</td>
<td>236</td>
</tr>
<tr>
<td>Mean age (range), years</td>
<td>31 (5–69)</td>
<td>31 (5–70)</td>
</tr>
<tr>
<td>Sex (n[%])</td>
<td>Male 109 (46)</td>
<td>108 (46)</td>
</tr>
<tr>
<td>Female</td>
<td>128 (54)</td>
<td>128 (54)</td>
</tr>
<tr>
<td>Marker of viral replication [male/female, n(%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male 7/10 (70)</td>
<td>10/10 (70)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>21/28 (75)</td>
<td>21/28 (75)</td>
</tr>
<tr>
<td>Marker of viral replication [males/females, n(%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male 17/17 (100)</td>
<td>17/17 (100)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>12/12 (100)</td>
<td>12/12 (100)</td>
</tr>
<tr>
<td>HBV vaccination (%)[a]</td>
<td>Unknown 2 (1)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>Yes</td>
<td>17 (7)</td>
<td>104 (44)</td>
</tr>
<tr>
<td>No</td>
<td>218 (92)</td>
<td>128 (56)</td>
</tr>
<tr>
<td>Village (%)[a]</td>
<td>Keneba 89 (38)</td>
<td>123 (52)</td>
</tr>
<tr>
<td>Manduar</td>
<td>95 (40)</td>
<td>66 (28)</td>
</tr>
<tr>
<td>Katong-Kunda</td>
<td>41 (17)</td>
<td>36 (15)</td>
</tr>
<tr>
<td>Other</td>
<td>12 (5)</td>
<td>11 (5)</td>
</tr>
</tbody>
</table>

Table 2. Subjects positive for serum R249S mutation by season (n/total [%]).

<table>
<thead>
<tr>
<th>Period</th>
<th>HBsAg negative</th>
<th>HBsAg positive</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>October–July</td>
<td>59/236 (25)</td>
<td>198/237 (45)</td>
<td>2.49 (1.68, 3.69)[a]</td>
</tr>
<tr>
<td>October–March (higher AFB1 exposure)</td>
<td>45/134 (34)</td>
<td>133/134 (99)</td>
<td>0.54 (0.34, 0.83)</td>
</tr>
<tr>
<td>April–July (lower AFB1 exposure)</td>
<td>13/102 (13)</td>
<td>63/103 (61)</td>
<td>10.9 (5.35, 22.34)</td>
</tr>
</tbody>
</table>

[a] ORs are adjusted for age and sex.

*Used as reference category for OR determination. **Adjusted for seasonality.
S_HBV778r (5'-gaggtataaaggactcaag-3'). HBV genotypes and subtypes were determined in collaboration with the Virological Department of Swedish Institute for Infectious Disease.

Statistical analysis. Values were expressed in numbers of R249S copies/mL serum, with resulting values between 0 and 63,800 copies/mL. For statistical analyses, values below the detection limit of 70 copies/mL were assigned a value of 70 copies/mL. Results showed large variations and were analyzed in three ways. First, we evaluated the distribution of values according to month of collection. Second, means ± SDs were calculated for groups of months corresponding to two periods of different levels of exposure to aflatoxin. Third, the number and percentage of subjects with values > 70 copies/mL were tabulated in relation with HBV serological status or the two periods of different aflatoxin exposure.

To analyze the impact of seasonal AFB1 exposure, we grouped results into two seasons: a) a higher AFB1 exposure period from October 2002 to March 2003, after the September–October harvest and including the dry season in February–March; and b) a lower AFB1 exposure period from April 2003 to July 2003, which included part of the June–August wet season. AFB1 accumulates in foodstuffs (principally grain grounds) in the Gambia (principal grain groundnuts) after harvest in September–October; levels of AFB1–albumin adducts (a biomarker of recent AFB1 exposure) have been shown to decrease from their highest levels in the February–March dry season to reach their lowest levels after the June–August wet season just before the next harvest (Turner et al. 2000; Wild et al. 2000). Statistical analyses were performed using STATA software (version 11.1; StataCorp LP, College Station, TX, USA) or by Student’s t-test to compare the average of R249S copies per milliliter in HBsAg-positive or -negative subjects depending on AFB1 exposure. Analysis involved standard unconditional logistic regression of the probability of R249S positivity using as explanatory variables HBsAg status and seasonality adjusted for sex and age.

Results

The characteristics of study participants are described in Table 1. Most individuals were recruited in Keneba and Manduar villages (HBsAg positive, 38% and 40%, respectively; HBsAg-negative, 52% and 28%). Almost all HBsAg-positive subjects had not been vaccinated (92%), whereas 44% of HBsAg-negative subjects had been vaccinated. Mean serum CFDNA concentrations (± SD) were not significantly different (p = 0.73) for HBsAg-positive subjects (0.11 ± 0.92 ng/μL) compared with HBsAg-negative subjects (0.09 ± 0.51 ng/μL).

A total of 164 subjects (106 HBsAg positive and 58 HBsAg negative) had R249S serum concentrations > 70 copies/mL and were considered positive for R249S (Table 2). HBsAg-positive subjects were more likely to be R249S positive than HBsAg-negative subjects (45% vs. 25%, respectively; odds ratio (OR) = 2.49; 95% confidence interval (CI): 1.68, 3.69).

Serum R249S concentrations were low for both HBsAg-negative and -positive subjects during the higher AFB1 exposure period (October–March) (Figure 1). However, during the lower AFB1 exposure period (April–July), both serum concentrations and the number of subjects positive for R249S appeared to increase in the HBsAg-negative group.

Mean (± SD) serum concentrations of R249S were similar for HBsAg-negative subjects during the higher (1.183 ± 5.230 copies/mL serum) and lower (400 ± 1,160 copies/mL serum) AFB1 exposure periods (p = 0.137) and for HBsAg-positive (1.183 ± 5.230 copies/mL serum) and HBsAg-negative (480 ± 1,030 copies/mL serum) subjects (p = 0.128) during the higher AFB1 exposure period (Figure 2). In contrast, the mean serum concentration of R249S in HBsAg-positive subjects during the lower AFB1 exposure period (5,690 ± 11,300 copies/mL serum) was significantly higher than in HBsAg-negative subjects during the same period (p < 0.001) and HBsAg-positive subjects during the higher AFB1 exposure period (p < 0.001). We also observed this seasonal variation when the data were analyzed qualitatively (Table 2). The proportion of R249S-positive samples was highest among HBsAg-positive subjects during the lower AFB1 exposure period (April–July) and lowest among HBsAg-negative subjects during the same period (61% and 13%, respectively; OR = 10.9; 95% CI: 5.35, 22.34) (Table 2). In contrast, the proportions of R249S-positive samples were comparable between HBsAg-positive and -negative subjects during the higher AFB1 exposure period (October–March; 32% and 34%, respectively; OR = 0.94; 95% CI: 0.56, 1.57). Thus, in HBsAg-positive subjects both the levels of
R249S in serum (Figure 2) and R249S positivity (Table 2) peaked several months after the previously reported high AFB1 exposure period in January–February. In HBsAg-positive subjects, 61% were R249S positive during the lower AFB1 exposure period compared with 32% R249S positive during the higher AFB1 exposure period (OR = 3.59; 95% CI: 2.05, 6.30) (Table 3).

Among HBsAg-positive subjects, 45% were R249S positive, 8% were positive for the 1762T/1764A HBV mutation, and 4% were positive for both R249S and 1762T/1764A (Table 4). Subjects who had both R249S and 1762T/1764A were an average of 5–11 years younger than other subjects (mean age [range] 26 (7–49) years, compared with 31 (7–65) years for R249S positive only, 37 (20–61) years for 1762T/1764A positive only, and 31 (5–68) years for negative for both mutations); however, this difference was not statistically significant (p = 0.08). Serum R249S positivity did not vary significantly according to 1762T/1764A status (Table 4), village of origin, sex, vaccination status, HBBeAg status, or serum concentration of viral DNA (data not shown).

HBBeAg status and presence of detectable HBV DNA also showed seasonal variation. Among HBsAg-positive subjects, the proportion that were HBBeAg positive (indicating active viral replication) was highest during April–June, whereas the proportion with undetectable HBV DNA decreased to <10% between March and May, consistent with a seasonal peak in viral replication at the transition during high and low AFB1 exposure periods, slightly ahead of the observed peak of R249S DNA (Figure 3). The seasonal patterns of R249S, HBBeAg, and HBV DNA were not associated with obvious differences in total serum CFDNA concentrations (data not shown).

To gain insight into the viral factors that may affect the occurrence of R249S in serum, we sequenced the entire HBV genome of 119 subjects. To further determine whether the presence or level of R249S may vary according to particular subgroups, we subdivided subjects with sequences belonging to genotype E into five groups based on sequence similarities. Three distinct S gene sequences were found: one in 8 individuals (group 1), another in 17 individuals (group 2), and the third in 11 individuals (group 3). Group 4 comprised 12 subjects with closely related sequences, and group 5 comprised all other subjects (42) with unique S gene sequences. We found no significant difference between levels of R249S or presence of 1762T/1764A double mutation and S sequence groups after adjustment for seasonality (data not shown).

Discussion

We used a sensitive and quantitative method to detect 7TP3 R249S in the serum of individuals from a rural area of the Gambia with no evidence of chronic liver disease. We obtained serum samples in a cross-sectional survey performed in 2002–2003 in three villages where consumption of AFB1-contaminated foodstuff and individual variations in exposure to AFB1 have been previously documented (Wild et al. 2000). Two matched groups of subjects were tested: subjects positive for HBsAg (chronic HBV carriers) and control subjects seronegative for HBsAg. We detected R249S more frequently in HBV carriers (45%) than in controls (25%; OR = 2.49; 95% CI: 1.68, 3.69). In both groups, we observed seasonal variations in the proportion of subjects positive for R249S and in the average concentration of serum R249S DNA. Our results showed that: a) R249S was detectable in CFDNA of a significant proportion of subjects with no symptoms of chronic liver disease; b) R249S positivity was associated with HBV carrier status; and c) the presence and levels of R249S in CFDNA varied according to season.

In the Gambia, groundnut culture occupies >50% of harvested land (Department of Agricultural Services, the Gambia 2008). Groundnuts are the main source of exposure to aflatoxins and are consumed seasonally according to availability. AFB1 accumulates in foodstuffs after harvest in September–October and peaks in January–February, when the

---

**Table 3. Subjects positive for serum R249S mutation by HBsAg status [n/total (%)].**

<table>
<thead>
<tr>
<th>HBsAg</th>
<th>October–Marcha</th>
<th>April–July</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>45/134 (34)</td>
<td>13/102 (13)</td>
<td>0.30 (0.15, 0.61)</td>
</tr>
<tr>
<td>Positive</td>
<td>43/134 (32)</td>
<td>63/103 (61)</td>
<td>2.59 (2.05, 6.30)</td>
</tr>
</tbody>
</table>

ORs are adjusted for age and sex.

*aUsed as reference category for OR determination.

---

**Table 4. HBsAg-positive subjects positive for serum R249S mutation and/or 1762T/1764A double mutation [n/ (%)].**

<table>
<thead>
<tr>
<th>1762T/1764A mutation</th>
<th>No</th>
<th>Yes</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1762T/1764A mutation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>122 (51)</td>
<td>9 (4)</td>
<td>131 (59)</td>
</tr>
<tr>
<td>Yes</td>
<td>96 (41)</td>
<td>10 (4)</td>
<td>106 (45)</td>
</tr>
<tr>
<td>Total</td>
<td>218 (92)</td>
<td>19 (8)</td>
<td>237 (100)</td>
</tr>
</tbody>
</table>

For 1762T/1764A compared with R249S mutation status, $\chi^2 = 5.222; p = 0.470$.

---

**Figure 3. Seasonal variation of HBsAg positivity (HBsAg+) and HBV DNA negativity (HBV−) in HBsAg-positive subjects.** The number of individuals sampled each month are given. The value for November 2002 was not considered because the sample size was too small.
availability of stored groundnuts and the relative lack of other crops lead to significantly higher exposure than during other months. We did not measure aflatoxin exposure in the present study, but the seasonality of exposure is well documented in adults (Wild et al. 1992) and children (Turner et al. 2000) in the Gambia. In addition, Wild et al. (2000) reported seasonal variation in AFB₁–albumin adduct levels in a longitudinal study of residents in the same villages included in the present study, with the highest level in February–March (83.2 pg AFB₁-lysine/mg albumin; 95% CI: 53.3, 130.8) and the lowest levels in June–July (34.9 pg AFB₁-lysine/mg albumin; 95% CI: 28.5, 42.8). In non-vaccinated children 3–4 years of age from four geographical areas representative of the Gambia as a whole, AFB₁–albumin adducts were detected in all samples (range, 2.2–459 pg AFB₁-lysine/mg albumin), and levels were twice as high in November–March as in April–July (lower AFB₁ exposure period) than during October–March (34% ; higher exposure than during other months). However, traces of 2.45 pg AFB₁-lysine/mg albumin, respectively). Adduct levels were significantly higher in acutely and chronically HBV-infected children, suggesting that infection may modulate AFB₁ metabolism and DNA adduct formation (Wild et al. 2000).

In the present study, we took this research one step further by showing evidence of seasonal variation in the R249S TP53 mutation in CFDNA. This mutation is a consequence of the formation of AFB₁-DNA adducts (Besaratinia et al. 2009; Gouas et al. 2009). In HBV-negative subjects, temporal patterns were similar to those described for AFB₁–albumin adducts, with a lower proportion positive for R249S (13%) among those surveyed during April–July (lower AFB₁ exposure period) than during October–March (34%; higher AFB₁ exposure period; OR = 0.30; 95% CI: 0.15, 0.61). In contrast, we observed the opposite pattern in HBV carriers, with a greater proportion positive for R249S (61%) among those surveyed during April–July than during October–March (32%; OR = 3.59; 95% CI: 2.05, 6.30). Furthermore, the average concentration of R249S in serum was higher in subjects surveyed in April–July than in those sampled in October–March (p < 0.001). The process of addition and fixation of mutation and persistence of the mutation require several steps, which could partly explain the apparent lag between increased detection of AFB₁–albumin adducts (a short-term biomarker of exposure to aflatoxin) and increased detection of R249S in CFDNA (which may occur days or weeks after exposure). However, taking these steps into account does not explain the observed difference between controls and HBsAg-positive subjects. Therefore, our results suggest that the dynamics and persistence of R249S mutations may be different in HBV carriers than in controls, consistent with an interaction between chronic HBV infection and R249S formation, amplification, and persistence.

Positivity for HBsAg and presence of detectable HBV DNA in the serum also showed seasonal variations, with a peak at the transition during periods of high and low AFB₁ exposure. Seasonal reactivation of HBV might induce damage to hepatocytes and increase R249S release into the bloodstream in the weeks that follow reactivation. Seasonal hepatitis flare was described by Zhang et al. (2006) in a longitudinal study of 2,238 subjects from the area of Guangzhou, China. In that study, acute exacerbation of hepatitis peaked during spring (March–May), and HBeAg seroconversion peaked during summer (June–August). So far, there is no report on seasonal patterns of HBV infection in West Africa.

To gain further insights on the role of HBV, we analyzed double mutations in the HBV X gene (1762T/1764A) that have been associated with infection severity. A recent prospective study in Thailand showed that chronic HBV carriers with 1762T/1764A double mutations had an elevated risk of HCC (OR = 2.47; 95% CI: 1.04, 5.85) (Kuang et al. 2005). In the Gambia, we observed this double mutation in some chronic carriers who were asymptomatic for chronic liver disease, and in most HCC or liver cirrhosis cases (Mendy et al. 2008). In the present series, we found 1762T/1764A double mutations in 19 of 237 HBV carriers (8%), with no association with R249S positivity. Sequencing of the entire S gene in 99 carriers detected genotype E in 95 cases and genotype A in four cases. We found no association between subgroups of genotype E and R249S positivity.

Although there are no data on the stability of R249S in serum, its presence at detectable levels may require the release of DNA from a large number of cells harboring mutant DNA. The limited data available on serum DNA turnover suggest a very short half-life. In a study on fetal DNA in maternal plasma after delivery, Lo et al. (1999) estimated the mean half-life of circulating fetal DNA to be 16.3 min (range, 4–30 min). In another study evaluating the persistence of Epstein-Barr virus DNA in plasma after surgery for nasopharyngeal cancer, Chan et al. (2008) found a half-life of 139 min. Thus, the lag time of several months observed between the peak of AFB₁ exposure and the peak of R249S serum levels in HBV carriers does not support that the release of R249S is the end product of mutagenesis and a rapid clearance of cells containing an R249S mutation. If this were the case, R249S DNA would occur in the bloodstream almost simultaneously to peak AFB₁ exposure, a scenario seen in controls but not in HBV carriers. Thus, our results suggest that, in carriers, an interaction between AFB₁ exposure and HBV might enhance the occurrence, persistence, and/or clonal expansion of cells with R249S mutations. In contrast, in controls, the formation and release of R249S were consistent with the seasonal pattern of exposure to AFB₁, which suggests that the presence of R249S in the serum of subjects with no detectable neoplastic liver lesion may serve as a biomarker of mutagenesis following dietary aflatoxin exposure. TP53 mutation load was associated with smoking dose and duration in a case–control study of lung cancer (Hagiwara et al. 2006), supporting the fact that mutations in CFDNA may serve as reporters of diverse forms of carcinogenic exposures.

In a previous case–control study of HCC in the Gambia (Lleonart et al. 2005), we showed that high plasma concentrations of R249S (> 10,000 copies/mL) were associated with HCC status (OR = 20; 95% CI: 5.6, 69.0). However, traces of R249S (< 2,500 copies) were found in many controls. In combination with our findings in the present study, results suggest that R249S in CFDNA may constitute a biomarker of exposure or a predictor of liver cancer, depending upon levels and temporal variation, such that low, transient plasma concentrations may reflect seasonal exposure to AFB₁, whereas high and sustained plasma concentrations may indicate the presence of a developing cancer lesion. Prospective studies on chronic HBV carriers in China have shown that R249S concentrations could be detected before HCC diagnosis, with a tendency to increase in R249S proportion with decreasing time to diagnosis (Jackson et al. 2003; Szyman ska et al. 2009). Further studies are needed to determine quantitative serum R249S thresholds as well as time-dependent variations that may distinguish subjects with ongoing AFB₁ exposure from subjects with liver lesions progressing toward HCC.

Conclusion

The presence and amount of TP53 R249S mutation in serum varied among survey subjects according to season, with a pattern that was distinct from the well-known seasonal variations in exposure to aflatoxin. Seasonal patterns differed between HBV carriers and noncarriers, suggesting a synergistic effect between HBV and aflatoxin exposure. This is the first demonstration of such an effect, in which being an HBV carrier appears to have a profound impact on the persistence of mutations induced by aflatoxin. This study also demonstrates that, in this particular exposure context, levels of R249S vary seasonally, suggesting that this mutation occurs multiple times in HBV carriers. A longitudinal study on HBV carriers exposed to aflatoxin is needed to determine how these variations influence the risk of developing chronic liver disease and, ultimately, HCC.
Naphthalene and Styrene. IARC Monogr Eval Carcinog Risk Hum 82:1–56.


