Ser-249 p53 Mutations in Plasma DNA of Patients With Hepatocellular Carcinoma From The Gambia


**Background:** A selective mutation, an arginine-to-serine substitution in codon 249, of the p53 gene has been identified as a “hotspot” mutation in hepatocellular carcinoma (HCC). This mutation occurs in populations that are exposed to aflatoxins and have a high prevalence of hepatitis B virus carriers. We evaluated whether this mutation could be detected in cell-free DNA isolated from the plasma of subjects from The Gambia to detect this mutation that is strongly associated with HCC. **Methods:** Fifty-three patients with HCC, 13 patients with cirrhosis, and 53 control subjects were prospectively recruited from The Gambia. Sixty patients, of non-African origin, with various liver pathologies were also selected from France. DNA was extracted and purified from 200-µL aliquots of plasma. The Ser-249 p53 mutation was detected by restriction endonuclease digestion of polymerase chain reaction products from exon 7 and was confirmed by direct sequencing of the amplified DNA. **Results:** The Ser-249 p53 mutation was detected in plasma DNA from 19 (36%) of the 53 patients with HCC, two (15%) of the 13 patients with cirrhosis, and three (6%) of the 53 control subjects. This mutation was not detected in any plasma DNA from the European patients. The adjusted odds ratio for having the mutation was 16.4 (95% confidence interval = 3.0–90.5) for patients with HCC compared with the control subjects. **Conclusion:** The Ser-249 p53 mutation in plasma DNA is strongly associated with HCC in Gambian patients. This mutation was also detected at a much lower prevalence in plasma DNA from Gambian patients with cirrhosis and in Gambian control subjects, findings that may lead to the earlier detection of HCC. Use of the Ser-249 p53 mutation should facilitate further molecular epidemiologic studies on the development of HCC. [J Natl Cancer Inst 2000;92:148–53]

**Materials and Methods**

**Subjects and Specimens**

Incident cases of HCC and cirrhosis were identified from liver disease referral clinics at each of the three tertiary referral hospitals in The Gambia (i.e., Royal Victoria Hospital, Medical Research Council Hospital, and Bansang Hospital). Samples from The Gambia analyzed in this study were from subjects sequentially recruited from September 1997 through June 1998 into an ongoing case-control study of HCC. We amplified DNA from 142 (92%) of 155 subjects enrolled during this period; we could not amplify DNA from 13 subjects. Evaluation of subjects included a clinical examination, ultrasonography, collection of biologic specimens, and a structured interview.

For inclusion in the study, a patient had to have compatible clinical and ultrasonographic findings and serum α-fetoprotein levels of 100 ng/mL or more. Twenty-three patients with HCC, who were initially recruited into the study, were excluded because their levels of α-fetoprotein were less than 100 ng/mL. Analyses performed with and without inclusion of these subjects were similar. The results presented include only patients who met the case definition of HCC, for a total of 119 study subjects (53 patients with HCC, 13 patients with cirrhosis, and 53 control subjects). Patients with cirrhosis were included as an additional referent group, in addition to healthy control subjects, to evaluate factors associated with the progression to HCC. The diagnosis of cirrhosis was based on compatible clinical history and ultrasonographic patterns characteristic of cirrhosis, without any focal lesions suggestive of HCC. A minority of patients with liver disease also had histologic confirmation. Control subjects were individuals with no history or clinical findings suggestive of liver disease, who were recruited from the outpatient clinic of one of the three tertiary referral hospitals. Control subjects were frequency matched to case patients with HCC by age (within 10-year groupings), sex, and recruitment site. Local and international ethical and scientific review committees approved the study protocol, and informed consent was obtained.

**Affiliations of authors:** G. D. Kirk, International Agency for Research on Cancer, Banjul, The Gambia, and Lyon, France, and Viral Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD; A.-M. Camus-Randon, P. Hainaut, R. Montesano, International Agency for Research on Cancer, Banjul and Lyon; M. Mendy, Medical Research Council Laboratories, Banjul; J. J. Goedert, Viral Epidemiology Branch, National Cancer Institute; P. Merle, C. Trépo, INSERM Unité 271, Lyon; C. Bréchot, Hepatology Unit, Necker Hospital, Paris, France.

**Correspondence to:** Ruggero Montesano, M.D., Ph.D., Unit of Mechanisms of Carcinogenesis, International Agency for Research on Cancer, 150 cours Albert-Thomas, 69372 Lyon Cedex 08, France (e-mail: montesano@iarc.fr).

See “Notes” following “References.”

© Oxford University Press
was obtained from each participant before inclusion in the study.

To compare West African subjects with subjects from areas with lower exposure to aflatoxin and HBV, we evaluated plasma and serum samples obtained from 60 non-African patients from Europe (Hotel Dieu Hospital [Lyon, France] and Necker Hospital [Paris, France]). Subjects included 50 patients with HCC (21 associated with HBV, 17 with hepatitis C virus, seven with alcohol, and five with unknown status) and 10 patients with cirrhosis (three associated with HBV, four with hepatitis C virus, and seven with alcohol [some patients with more than one association]). The samples were sent to the laboratory of the International Agency for Research on Cancer (IARC) and were analyzed in the same manner as the samples from The Gambia.

DNA Extraction and Polymerase Chain Reaction

Blood specimens anticoagulated with EDTA were processed immediately after collection and stored at −70 °C at the Medical Research Council Laboratories, The Gambia, until subsequent testing. α-Fetoprotein and HBV serologic testing was performed with standard laboratory kits. A 500-μL aliquot of plasma was shipped on dry ice to IARC laboratories (Lyon, France) to test for the Ser-249 p53 mutation. DNA was extracted from 200 μL of plasma with a QiAmp tissue kit (Qiagen, Hilden, Germany). The purified DNA was eluted from the QuiAmp silica column with nuclease-free water (Promega Corp., Madison, WI).

Ten microliters of DNA eluate was used to amplify exon 7 of the p53 gene with primers located in the introns flanking exon 7, as described by Lehman et al. (19). The following primers (Genset, Paris-France) were used: p1 (sense), 5'-CTTGGCCAACAG-GTCTCCCCAA-3'; p2 (antisense), 5'-AGGGGT-CAGCGGCAAGCAGA-3'.

The 50-μL reaction mixture contained 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 1.5 mM MgCl₂, all four deoxyribonucleoside triphosphates (each at 0.2 mM; Promega Corp.), 0.2 μM of each primer, and 2.5 U of Platinum Taq DNA polymerase (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). After an incubation (94 °C for 2 minutes), 35 cycles (94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds) were performed and followed by a final 5-minute extension at 72 °C in a thermal cycler (gene Amp PCR system, 9600; The Perkin-Elmer Corp., Foster City, CA). The amplification products (254 base pairs [bp]) were visualized by staining with ethidium bromide after electrophoresis on 3% agarose gel.

In six samples, the polymerase chain reaction (PCR) products were not clearly detected, and a second amplification reaction was performed with the following nested primers: p3 (sense), 5'-AGGCG-GCACTGGCCTTCTTT-3'; p4 (antisense), 5'-TGTGCAAGGGTGCCAAAGTGGC-3'. This reaction mixture contained 5 μL of the first PCR products, and 25 cycles (as in the first PCR) were performed. The same nested PCR protocol was also used to generate enough material for DNA sequence analysis (see below). All sets of PCRs included negative control samples (containing no template DNA). For avoidance of PCR contaminant artifacts, routine precautions included performing DNA extraction, PCR assembly, and the PCR in separate rooms and using barrier tips at all stages of the procedure.

Mutation Detection by Restriction Endonuclease Analysis

PCR products (10 μL) were digested with restriction endonuclease HaeIII (Boehringer Mannheim GmbH, Mannheim, Germany), according to the manufacturer’s instructions. This enzyme cleaves a GG/CC sequence between codon 249 and codon 250 to generate two fragments of 92 bp and 66 bp (plus several small fragments) from the 254-bp product of the first PCR. The presence of a mutation in codon 249 or codon 250 results in an uncleaved 158-bp fragment. These fragments were separated and identified on 3% agarose gel stained with ethidium bromide (Fig. 1). The absence of the 254-bp band (full-length PCR products) provides a control for complete digestion of the PCR product. Restriction endonuclease assays included both positive (DNA with a Ser-249 p53 mutation isolated from a patient with liver cancer) and negative (wild-type DNA) controls. All reactions were carried out in duplicate. As a further control, five positive and five negative assays were selected at random and submitted to a second, independent analysis, including DNA extracts, PCR, and restriction endonuclease digestion. Concordant results were obtained in each case.

For the determination of the relative sensitivity of this method to detect a mutation, a control DNA sample containing a mutation at codon 249 was serially diluted 1:2 up to 1:256 in wild-type DNA. The mixture was amplified by PCR as described above and analyzed by restriction endonuclease digestion. The presence of the uncleaved 158-bp fragment was clearly visible at ratios of mutant to wild-type DNA of 1:64.

The 158-bp product, not cleaved between codon 249 and codon 250, was excised from agarose gels and further amplified with nested PCR primers P3 and P4, as described above. The products of this second PCR were purified with QIA quick-spin columns (Qiagen), and 0.25 μg of DNA (exon 7, codons 225–260) was analyzed by automated DNA sequencing (sequencer 377; The Perkin-Elmer Corp.) with the use of the dRhodamine terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) (Fig. 1).

Statistical Analysis

Frequency tables of independent variables and Ser-249 p53 mutations, as a dichotomous variable, were evaluated for statistical significance by Pearson’s χ² test and Fisher’s exact test. For the estimation of the risk of mutations among the different groups in the study while adjustment was made for potential confounders (including age, sex, recruitment site, and hepatitis B surface antigen [HBsAg] status, a marker of chronic infection with HBV), a multivariable logistic regression analysis was performed to estimate odds ratios (ORs) and 95% confidence intervals (CIs). In a separate stratified analysis, only data from patients with HCC were evaluated by similar methods to examine factors associated with Ser-249 p53 mutation-positive HCC compared with Ser-249 p53 mutation-negative HCC. All analyses were performed with STATA version 5.0 (Stata Corporation, College Station, TX) and EPI-Info version 6.0 (Centers for Disease Control and Prevention, Atlanta, GA; and World Health Organization).
RESULTS

The characteristics of the 179 subjects evaluated from The Gambia and Europe are shown in Table 1. The age and sex distributions of Gambian patients with HCC and Gambian control subjects were similar. Among Gambian subjects, patients with cirrhosis were markedly younger than patients with HCC but had a similar sex ratio. Sixty-four percent of Gambian patients with HCC, 54% of Gambian patients with cirrhosis, and 17% of Gambian control subjects were chronic carriers of HBV \( (P < 0.001 \) for difference between groups). As expected, the age distribution of European patients with HCC or with cirrhosis was considerably older, and the prevalence of HBV carriers was lower than that in The Gambia. The age difference between European patients with cirrhosis and European patients with HCC and the sex ratios observed among European subjects were similar, although less pronounced, compared with those observed among Gambian subjects.

A Ser-249 p53 mutation was present in 19 of 53 patients with HCC, two of 13 patients with cirrhosis, and three of 53 control subjects (Table 2), giving a prevalence of 36%, 15%, and 6%, respectively \( (P < 0.001 \) for difference between groups). In univariate analysis stratified by subject group, there was no statistically significant difference in mutation prevalence by age, recruitment site, or HBsAg status. However, females were statistically significantly more likely to have the Ser-249 p53 mutation than males among both patients with HCC \( (P = 0.012 \) and control subjects \( (P = 0.029 \). No Ser-249 p53 mutation was observed in 60 sera/plasma samples from European patients with either HCC or cirrhosis.

In multivariable analysis adjusting for age, sex, recruitment site, and HBsAg status, patients with HCC were 16-fold more likely to carry the Ser-249 p53 mutation \( (OR = 16.4; 95\% \text{ CI } 3.0–90.5 \) compared with control subjects (Table 2). Patients with cirrhosis also had an increased risk compared with control subjects \( (OR = 3.2 \) but with a wide 95% CI that included unity \( (95\% \text{ CI } 0.4–26.1 \). In this model, female gender was statistically significantly associated with Ser-249 p53 mutations, with an adjusted OR of 4.6 \( (95\% \text{ CI } 1.3–16.4 \) compared with males.

When the analysis among only patients with HCC was stratified by mutation status, no difference in Ser-249 p53 mutation prevalence was observed by age or HBsAg status (Table 3). Females were again overrepresented in the Ser-249 p53 mutation-positive group; however, after adjustment for age, recruitment site, and HBsAg status, the strength of the association was of borderline statistical significance \( (P = 0.053 \).

DISCUSSION

We report the presence of Ser-249 p53 mutations in DNA isolated from the plasma of individuals from a West African population at high risk for HCC, exposure to the carcinogen AFB\(_1\), and chronic HBV infection. This “hotspot” mutation was detected in 36% of patients with HCC (Table 2), with a much lower prevalence in patients with cirrhosis and control subjects. No such mutation was present in the plasma of 60 European patients with HCC or cirrhosis.

Circulating plasma DNA can be retrieved and analyzed for genetic alterations present in the original tumor. In some studies \( (16–18,20,21 \), various genetic alterations, such as loss of heterozygosity or K-ras mutations, have been identified in the plasma or sera and tumor tissue from patients with cancer of the lung, head and neck, kidney, colon, and pancreas. These observations and the absence or rarity of alterations in control subjects provide strong evidence that the altered DNA, circulating in the plasma or sera of the patient, originates from the tumor. Although knowledge of the underlying mechanisms of this circulating DNA is still limited, there is some evidence that the DNA is released from the tumor as a
glyconucleoprotein complex that may protect it from degradation by nucleases. It remains unclear whether release of tumor DNA into plasma is associated with tumor necrosis, apoptotic cell death, or other selective cellular processes (22–24).

Despite these uncertainties, the data that we present on Ser-249 p53 mutations are consistent with the premise that plasma or serum may be used as a source of tumor-specific DNA. In addition, the data show that this mutation is present in the plasma DNA of patients with HCC from a country with high exposure to AFB1, but not from Europe. Among 10,385 p53 mutations described in human cancers and compiled in the IARC p53 mutation database (http://www.iarc.fr/p53/) (25), a total of 197 are Ser-249 p53 mutation, 130 (66%) of which occur in patients with HCC originating from regions with a high incidence of HCC and high exposure to AFB1. Twenty-five percent of the Ser-249 p53 mutations were from lung cancers. The presence of this mutation in patients with HCC who are from Europe, the United States, Japan, and Australia is extremely low; only three mutations (one in Europe and two in Japan) were identified among 664 patients with HCC analyzed (6). These data provide strong, albeit indirect, evidence that the DNA containing the Ser-249 p53 mutation originates from the liver. In most developing countries, autopsies or biopsies for HCC are rarely performed; likewise, in this study, we did not have access to tumor tissue from the patients who donated the plasma samples. This is an important limitation of the present study. Collection of paired tumor and plasma samples for confirmation of concordant mutation status is under way in The Gambia. In neighboring Senegal, the prevalence of Ser-249 p53 mutations was reported to be approximately 67% in a series of 15 patients with HCC (26). The limited data available in The Gambia on the Ser-249 p53 mutation in HCC tumors are consistent with these findings (data not shown). Thus, we estimate that around 70% of patients with HCC who have a detectable Ser-249 p53 mutation in their tumor might also have detectable amounts of mutated DNA in their plasma.

Detection of the Ser-249 p53 mutation in circulating DNA depends on the ability of the method used to detect mutant DNA in a background of wild-type p53 DNA, which is estimated at 1:64 in the present study, based on serial dilution experiments. Preliminary results from the potentially more sensitive mass spectrometry analysis (27,28) of PCR products obtained with different primers also demonstrated the presence of Ser-249 p53 mutation in plasma samples from The Gambia (Friesen M; personal communication). By increasing the sensitivity of this method, DNA with a Ser-249 p53 mutation may be detected in more individuals.

It is interesting that, in this study, Ser-249 p53 mutations were detected not only in patients with HCC but also in patients with cirrhosis (15%) and a small proportion of control subjects (6%). The Ser-249 p53 mutation has been reported previously in nonmalignant liver tissue at frequencies that paralleled the levels of exposure to AFB1, suggesting that this mutation could be an early genetic event in hepatocarcinogenesis (29). Detection of the Ser-249 p53 mutation in DNA may thus be useful as a marker of neoplastic development. Alternatively, the presence of the mutation in healthy subjects may reflect chronic exposure to high levels of AFB1. The Ser-249 p53 mutation in plasma DNA could be a marker of disease (an early neoplastic effect), exposure (a biologic effect of aflatoxin exposure), or both. Consequently, detection of this mutation may perhaps identify high-risk individuals who could benefit from more intensive evaluation that could detect HCC earlier.

There is substantial evidence that the Ser-249 p53 mutation present in HCC is directly attributable to DNA damage induced by exposure to dietary AFB1 (6,30). This proposal is based on the following observations: (see (7)) 1) AFB1 is highly mutagenic and carcinogenic; 2) DNA lesions induced by AFB1 at guanine residues in double-stranded DNA are by no means random and are dependent on flanking nucleotide sequences (31,32); 3) guanine-to-thymine transversion is the prevalent base change in mutations induced by AFB1 (33,34); 4) the AFB1 metabolite binds specifically to the third nucleotide (AGG) in codon 249 (29,35); and 5) the Ser-249 p53 mutation is present in nontumorous tissues in patients from populations at high risk for HCC (29). One recent article (36) that questioned the association between Ser-249 p53 mutations and exposure to AFB1 was based on a highly selective literature review (see (6,7)) and data from an in vitro system that does not mimic the in vivo selective advantage of cells containing this specific mutation (see (7)). At the same time, the Ser-249 p53 mutation has been reported in cultured human cells treated with oxygen radicals (37).

Among patients with HCC, we did not observe any differences in chronic infection with HBV (based on HBsAg status) between those patients with HCC who were positive for the Ser-249 p53 mutation in plasma DNA and patients with HCC who were negative for this mutation (Table 3). Therefore, the highly statistically significant increased risk of HCC associated with Ser-249 p53 mutations in plasma DNA may be, at least in The Gambia, independent of the effects of chronic HBV infection. Geographic and ecologic studies of HCC have been limited by the fact that regions with a high incidence of HCC frequently have a population that is simultaneously exposed to AFB1 and HBV. Other studies have been limited by the short period of exposure reflected by urinary or serum aflatoxin biomarkers. Because of this complexity, there are at present limited epidemiologic data to permit an estimation of the cancer risk attributable to AFB1 exposure that is independent of HBV infection and the risk attributable to an interaction between these two factors (38). A recent prospective study of Chi-

<table>
<thead>
<tr>
<th>Ser-249 p53 mutation</th>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>34</td>
<td>19</td>
</tr>
<tr>
<td>Median age, y (range)</td>
<td>47.5 (23–87)</td>
<td>46 (19–80)</td>
</tr>
<tr>
<td>% male</td>
<td>31 (91)</td>
<td>12 (63)</td>
</tr>
<tr>
<td>No. of hepatitis B surface antigen-positive subjects (%)</td>
<td>23 (68)</td>
<td>11 (58)</td>
</tr>
</tbody>
</table>

*Two-sided P<.05. Other two-sided P values for comparisons between group were not statistically significant. In a model adjusting for age and hepatitis B surface antigen status, the sex difference between mutation-positive and mutation-negative case patients with hepatocellular carcinoma was of borderline statistical significance (two-sided P = .053).

Table 3. Comparison of Gambian patients with hepatocellular carcinoma who are negative or positive for the Ser-249 p53 mutation in plasma DNA
nese HBV carriers showed a threefold increased risk for HCC among individuals with detectable aflatoxin levels, as determined by urinary biomarker measurements on pooled urine samples (39,40). This observed effect of aflatoxin with HBV is similar to the independent effect but notably less than the multiplicative interaction associated with aflatoxin biomarkers observed in the original Chinese studies by Ross and colleagues (4,5). Likewise, our data are consistent with an independent causal association between AFB1 exposure, HBV carrier status, and the subsequent risk of HCC. The use of the Ser-249 p53 mutation detection in plasma or serum DNA as a marker of aflatoxin exposure, along with serologic markers of HBV status, may provide a better estimation of the risk attributable to these agents and a better understanding of their interaction in cancer development. Such studies will have a direct bearing on evaluating the effect of HBV vaccination in preventing HCC presently under way in The Gambia (41).

Despite the 4:1 preponderance of males among patients with HCC, females were consistently more likely to have the Ser-249 p53 mutation. At present, to our knowledge, no data are available to suggest that the prevalence of Ser-249 p53 mutations in HCC tumors might be higher in females than in males. Further studies are needed to confirm this observed sex difference (Table 3).

In addition to p53 mutations, other alterations have been reported in HCC in the genes controlling the transition from G1 to S phase of the cell cycle, such as amplification of the cyclin D1 gene (42) and inactivation of expression of the p16INK4a gene, resulting from hypermethylation of this gene (43). A recent study (18) reported a high prevalence of aberrant methylation of the p16 gene in serum DNA from patients with HCC. Somatic mutations in the β-catenin gene (44) and other genetic changes (45) are also frequently detected in HCC tumors. The possibility of detecting these genetic or epigenetic changes in blood specimens from patients with HCC should further elucidate the natural history of HCC and eventually assist in earlier detection and treatment of this cancer.

REFERENCES


NOTES

Supported in part by Public Health Service contract N02CP40521 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

We thank the patients and their families for their participation; the administrative and clinical staff of the Royal Victoria Hospital, Medical Research Council Hospital, and Bansang Hospital; the staff of the Medical Research Council Laboratories at Fajara and Basse, The Gambia; the Research Triangle Institute, Research Triangle Park, NC, for project management; and M. Wrisez for administrative support.

Manuscript received July 19, 1999; revised October 28, 1999; accepted November 10, 1999.