

## Ser-249 TP53 MUTATION IN TUMOUR AND PLASMA DNA OF HEPATOCELLULAR CARCINOMA PATIENTS FROM A HIGH INCIDENCE AREA IN THE GAMBIA, WEST AFRICA

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**Hepatocellular carcinoma (HCC) is frequent in areas of high exposure to aflatoxin and high prevalence of HBV infection, such as western Africa and south-east China. A selective mutation in TP53 (AGG→AGT at codon 249, Arg→Ser) has been identified as a hotspot in HCCs from such areas, reflecting DNA damage caused by aflatoxin metabolites. Recent studies have shown that circulating free DNA can be retrieved from human plasma, and it is hypothesised that plasma DNA may serve as a source for biomarkers of tumorigenic processes. In our study, we have determined the prevalence of Ser-249 mutation, using a PCR-restriction digestion method, with selective use of short oligonucleotide mass spectrometry analysis (SOMA), in a series of 29 biopsy specimens of HCC from The Gambia in West Africa. Overall, we identified the Ser-249 mutation in 35% (10/29) of the tumours. In parallel, we tested 17 plasma samples from HCC patients with matching tumour tissue. The 249 status concordance between tumour tissues and matched plasma was 88.5%. These results indicate that the Ser-249 mutation is common in HCC in The Gambia (35%), although a higher prevalence has been reported in other regions with high population exposure to aflatoxin (e.g., eastern China: >50%). Moreover, our studies indicate that plasma is a convenient source of liver tumour-derived DNA, thus holding promise for earlier detection and diagnosis of cancer.**

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**Key words:** Ser-249 TP53; hepatocellular carcinoma; plasma; circulating free DNA; the Gambia; aflatoxin

Hepatocellular carcinoma (HCC) is the most frequent form of liver cancer and is a major cause of death in subSaharan Africa and eastern Asia.<sup>1</sup> The primary aetiological factors in these regions are chronic infections by hepatitis viruses (mainly HBV) and dietary exposure to aflatoxins, a group of mycotoxins that are natural contaminants of the staple diet. These factors have a multiplicative effect on the risk of developing HCC.<sup>2</sup> In other regions of the world, such as the US and Europe, alcohol-induced liver injury is a major risk factor for HCC.

The pathogenesis of HCC involves multiple genetic alterations, affecting in particular genes involved in growth and cell cycle control. These genes include *p16/CDKN2A* (deletions, hypermethylation; in up to 50% of the cases), *β-Catenin* (mostly mutations in exon 3, 20–30%) *cyclins A* and *D* (overexpression, 20–30%) and *M6P/IGF2R* (mutations, 18–33%). Alterations of the *TGF-β* signalling pathway genes (*Smad 3*, *Smad 4*) have been described in a small proportion of the cases (up to 10%; for review, see references 3,4). As in many cancers, mutations in *TP53* are frequent but exhibit striking differences in prevalence and pattern, depending on the geographic area. In areas of low incidence of HCC, *TP53* mutations are found in up to 30% of the cases. These mutations are scattered along the DNA-binding domain of the gene, with no particular hotspot. In contrast, in areas of high incidence, *TP53* is mutated in over 50% of the cases, with a high proportion of a single missense mutation at codon 249, AGG to AGT, resulting in

the substitution of a serine for an arginine (Ser-249). This mutation represents 26% of all *TP53* mutations described to date in HCC and is rather uncommon in other cancers, with no tumour type having more than 3% of this alteration.<sup>5</sup> Ser-249 is by far the predominant mutation in areas of high HCC incidence and at the same time high aflatoxin exposure, like Mozambique,<sup>6</sup> Senegal<sup>7</sup> and Qidong county in China.<sup>8–10</sup> In contrast, the prevalence of Ser-249 is lower in other areas of China as well as in those African and Asiatic countries where the average levels of aflatoxin exposure are lower, such as Thailand.<sup>5,11</sup>

Aflatoxin B1 (AFB1) is actively metabolised in liver cells by several CYP450 enzymes (mainly 1A2 and 3A4). The primary DNA adduct of AFB1 is 8-9, dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin (AFB1-N7-Gua), which is naturally converted to 2 secondary lesions, an apurinic (AP) site and a relatively stable, AFB1-formamidopyrimidine (AFB1-FAPY) adduct. While both lesions can generate G to T transversions with an efficiency that depends upon local sequence context, recent evidence suggests that the most mutagenic lesion is the AFB1-FAPY adduct.<sup>12</sup> The sequence context of codon 249 (AGGCC) is consistent with a site of intermediate affinity for the formation of AFB1-induced lesions. Codon 249 is not the exclusive site of formation of promutagenic lesions by AFB1, and in experimental systems, the mutations have been reported at other codons.<sup>13–15</sup> The selection for mutations at codon 249 may be due to slower repair at this position, perhaps due to the interference of the HBx Ag of HBV with the host's repair system.<sup>16</sup> However, it is also likely that Ser-249 exerts specific pro-oncogenic effects on liver cells, resulting in its selection during the pathogenesis of HCC.<sup>17</sup>

Segments of free DNA are found in the plasma of all individuals at concentrations varying upon their health status. The concentration of circulating free DNA (CFDNA) is on average higher in cancer patients (180 ng/ml) than in healthy individuals (13 ng/ml).<sup>18</sup> However, there are wide inter-individual variations and it is

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unclear whether this DNA originates from lysis of tumour cells in the blood stream, necrosis and/or apoptosis of cancer cells in the tumour mass or active release of DNA by the tumour.<sup>19,20</sup> Yet, it has been shown that in cancer patients, CFDNA contains alterations in oncogenes and tumour suppressor genes, which is consistent with a significant portion of CFDNA originating from the tumour.<sup>20</sup> The concordance between genetic changes in CFDNA and in tumour cells varies according to tumour type and study design.<sup>19,20</sup>

In a recent case-control study of liver cancer from The Gambia, West Africa, we have detected Ser-249 mutation in the plasma of 36% of HCC patients (19/53 cases), 15% of individuals with liver cirrhosis (2/13) and 6% of healthy control subjects (3/53). No Ser-249 mutation was detected in CFDNA of European HCC patients with no exposure to AFB<sub>1</sub>.<sup>21</sup> In The Gambia, HCC is the most common cancer in males (ASR: 50/100,000/year) and the second most common after cervical cancer in females (25/100,000/year);<sup>22</sup> 15 to 20% of the general population in The Gambia are chronic HBV carriers<sup>23</sup> and 95% are exposed to high doses of aflatoxin B<sub>1</sub>, as shown by the high prevalence of AFB<sub>1</sub>-albumin adducts in the plasma.<sup>5,24,37</sup> In the current study from The Gambia, we did analysis of Ser-249 TP53 mutation in 29 HCC specimens and in 17 paired tumour-plasma specimens.

#### MATERIAL AND METHODS

##### *Patients and specimens*

In 1986, the International Agency for Research on Cancer (IARC, WHO) in cooperation with the Medical Research Council of UK and the Gambian Government initiated the Gambia Hepatitis Intervention Study (GHIS) in order to evaluate the efficacy of childhood HBV vaccination in childhood prevention of liver cancer.<sup>25</sup> In the context of the follow-up of this large intervention study, radiographic and pathological support for the diagnosis of HCC has been strengthened in The Gambia. Specimens evaluated for the current study included 36 liver samples obtained through routine clinical care and evaluated at the Royal Victoria Hospital Department of Pathology. Specimens from an additional 24 HCC cases were obtained as part of a case-control study on risk factors for HCC in The Gambia, supported by the US National Cancer Institute.<sup>21</sup> The criteria for the selection of the HCC cases and control participants have been described previously by Kirk *et al.*<sup>21</sup> Briefly, patients with clinical and ultrasonographic findings suggestive of HCC were identified at 3 referral hospitals in The Gambia (Royal Victoria Hospital, Banjul, MRC Clinical Services, Fajara and Bansang Hospital, Bansang). In the case-control study, 171 HCC cases underwent blood collection and were analysed for the presence of Ser-249 mutation in plasma DNA. Blood specimens were collected at the time of clinical examination prior to diagnosis, anti-coagulated with EDTA, processed immediately after collection and stored at  $-70^{\circ}\text{C}$ . An aliquot of plasma was shipped on dry ice to IARC.

Paraffin-fixed liver tissue samples (primarily needle biopsies) were available from the archived cases. Liver samples from selected HCC cases which underwent needle biopsy from the case-control study were fixed in buffered formalin and embedded in paraffin. Histological review of all liver specimens was performed by 3 independent pathologists in Banjul (O.S.) and in Lyon (P.T., J.Y.S.). In total, only 35 of the 60 biopsies of liver lesions obtained had sufficient material to be evaluated. Six additional specimens were excluded from analysis based on the pathologic diagnosis (liver haemangioma, 1 case; liver metastasis of unspecified primary tumour, 5 cases). The 29 remaining lesions were classified as hepatocellular carcinomas (HCC).

A clinical exemption was obtained from the NIH Office of Human Subjects Research for the analysis of archived liver specimens with identifying information removed. All case-control study participants signed an informed consent and the study was approved by Ethical Review Boards in The Gambia, NCI and at IARC.

##### *Viral status*

HBsAg was determined in the plasma as a marker of chronic HBV carriage by reverse passive hemagglutination assay (RPHA) (Murex Diagnostics Limited, Dartford, UK) with radioimmunoassay (RIA) testing of RPHA negative samples (Sorin Biomedica Diagnostics, Vercelli, Italy). Participants positive for HBsAg were tested for HBV "e" antigenemia (HBeAg) as a surrogate marker of active replication using an RIA kit (DiaSorin, Italy). HCV antibody status (anti-HCV) was determined by third generation ELISA (ORTHO Clinical Diagnostics, Neckargemund, Germany), and ELISA reactives were confirmed by recombinant immunoblot assay (HCV 3.0 SIA, CHIRON, Emeryville, CA). AFP was quantified by standard radiometric assay methods.

##### *Immunohistochemistry*

Paraffin sections (3  $\mu\text{m}$ ) were immunostained with a purified rabbit immunoglobulin against human p53 (CM1; NovoCastra, 1/1,000), recognising all isoforms of the protein. Immunostaining reactions were performed as described elsewhere.<sup>26</sup>

##### *DNA extractions*

DNA was extracted from 3  $\mu\text{m}$  unstained paraffin sections. The slides were deparaffined in xylene and ethanol, and tumour areas were scraped into 25–50  $\mu\text{l}$  of DNA extraction buffer (TE pH 9, proteinase K 0.1 mg/ml, 0.25% Nonidet P40), and incubated with proteinase K (20  $\mu\text{g}/\mu\text{l}$ ) for 3 to 5 days. DNA was also extracted from 100  $\mu\text{l}$  of plasma, using QIAamp<sup>®</sup> DNA Blood Mini Kit according to the manufacturer's blood and body fluid spin protocol (Qiagen, Chatsworth, CA). Purified DNA was eluted from the QIAamp Silica column with 2 volumes (2  $\times$  50  $\mu\text{l}$ ) of water (PCR-grade, Sigma Chemical Co., St. Louis, MO). DNA concentration was measured using Picogreen dsDNA quantitation kit (Molecular Probes, Eugene, OR). The fluorescence was read with a Fluoroskan Ascent FL fluorometer (Labsystems, Helsinki, Finland).

##### *PCR, restriction digestion and sequencing*

DNA (2–8  $\mu\text{l}$  of resuspended, purified material) was used for amplification of exon 7 of the *TP53* gene with 0.2  $\mu\text{M}$  primers (final concentration) flanking this exon (sense: 5'-CTTGCCA-CAGGTCTCCCCAA-3', antisense: 5'-AGGGGTCAGCGCA-AGCAGA-3'; Genset) using HotStarTaq polymerase (Qiagen). When necessary, a second PCR reaction was performed using nested primers (sense: 5'-AGGCGCACTGGCCTCATCTT-3', antisense: 5'-TGTGCAGGGTGGCAAGTGGC-3'; Genset). Both PCR reactions involved a 15 min HotStarTaq polymerase activation at 95°C, from 40 to 60 cycles of denaturation (94°C, 30 sec), primer annealing (60°C, 30 sec) and extension (72°C, 30 sec), followed by a final 5 min extension at 72°C. The size of the final PCR fragment was 177 bp. This extensive PCR protocol was necessary given the poor quality of DNA available for some of the specimens. In parallel with all reactions, blank controls were run to monitor for possible contamination.

PCR product (10  $\mu\text{l}$ ) was digested by HaeIII restriction endonuclease (Boehringer Mannheim Biochemicals, Indianapolis, IN), which cuts within a GGCC sequence encompassing codon 249 (AGG). Digestion of wild-type DNA generates 2 bands of 92 and 66 bp, whereas mutant material, in which the restriction site has been destroyed, yields a band of 158 bp. A second HaeIII restriction site present in the PCR product but located outside the coding area of exon 7 provided a positive control for digestion. Mutant, 158 bp fragments, visualised on 3% agarose gel stained with ethidium bromide, were cut out of the gel, reamplified (5  $\mu\text{l}$  of DNA, 30 cycles in conditions as described above), purified with a QIAquick PCR Purification Kit (Qiagen), and sequenced by automated, dideoxy sequencing (sequencer AbiPrism 3100, Perkin Elmer, Oak Brook, IL). All analyses were repeated at least twice.

For specimens yielding inconsistent results, additional analysis were performed using temporal temperature gradient electrophoresis (TTGE) and/or short oligonucleotide mass spectrometry analysis (SOMA).<sup>28</sup>

At all steps of the procedure, a series of controls without DNA template was used to rule out a contamination. Negative (wild-type genomic DNA) and positive (DNA of PLC/PRF/5 cells, ATCC, number CRL-8024, homozygous for Ser-249 mutation) controls were included in all restriction analyses. The sensitivity of the method was evaluated using serial dilutions of PLC/PRF/5 DNA. The restriction digestion could detect mutant material at a minimum rate of about 3% as compared to wild-type sequence.

Specimens appearing wild-type at digestion, were sequenced directly (from a third PCR product) to screen for possible other mutations in exon 7. Some tissue specimens were additionally screened for mutations in exon 5, 6 and 8 of *TP53* by direct sequencing.<sup>29,30</sup>

#### Statistical analysis

Liver specimens from both the archived collection and the case-control study were analysed together. Statistical significance in comparisons of the proportions of HCC tissue specimens by p53 immunohistochemistry, Ser-249 mutation and alpha-fetoprotein status was assessed through Pearson's chi-square test. Analysis of these outcomes by demographic or other tumour characteristics was also assessed by Pearson's chi-square test or Fisher's exact test where appropriate. All *p* values presented are 2-sided.

## RESULTS

### Patients and tumours

Demographic and histological characteristics of the 29 patients with confirmed HCC are listed in Table I. Interestingly, 9 of the 29 HCCs (31%) contained a predominant component of clear cells (Fig. 1a). Primary liver clear cell carcinoma (HCC-CC) is a cytological variant defined by WHO as "hepatocellular tumor predominantly or totally composed of clear cells" due to abundant glycogen, which does not stain with haematoxylin-eosin. The 20 other HCCs were classified according to Edmondson and Steiner

criteria (HCC E1 to E4). Serum alpha-fetoprotein (AFP) levels were available for 18 patients and were above 100 ng/ml in 15 of them. Out of 3 cases with AFP levels lower than 100 ng/ml, 2 were HCC-CC (2/9, 22%) and 1 was HCC Edmondson grade 3/4 (1/20, 5%, *p*=0.07). Ultrasonographic findings suggestive of cirrhosis were infrequent among study participants.

Data on HBV and HCV status were available for 19 patients (Table I). All patients were negative for HCV, whereas all but 1 were positive for HBV, further stressing the role of chronic HBV infections in the pathogenesis of HCC in The Gambia. Two patients (11 and 14) were positive for "e" antigen, a marker of active hepatitis. Mutant p53 protein often accumulates in cancer cells due to protein stabilisation. To determine whether mutation of *TP53* was correlated with accumulation, immunohistochemistry for p53 was positive (at least 10% of stained tumour cells) in 13 of 28 tumours analysed (Table I, Fig. 1c). Seven tumours exhibited staining in over 50% of tumour cells. Interestingly, 5 of 9 (56%) HCC-CCs were positive for p53 staining, compared to 3/11 (27%, *p*=0.10) HCC E1-E2 and 5/8 (63%, *p*=0.61) HCC E3-E4. For one patient (4), 4 different zones of the same tumour were analysed separately. All zones were classified as clear-cell HCC and exhibited 20–50% p53 immunostaining positivity except 1 zone which contained only 10% positive cells.

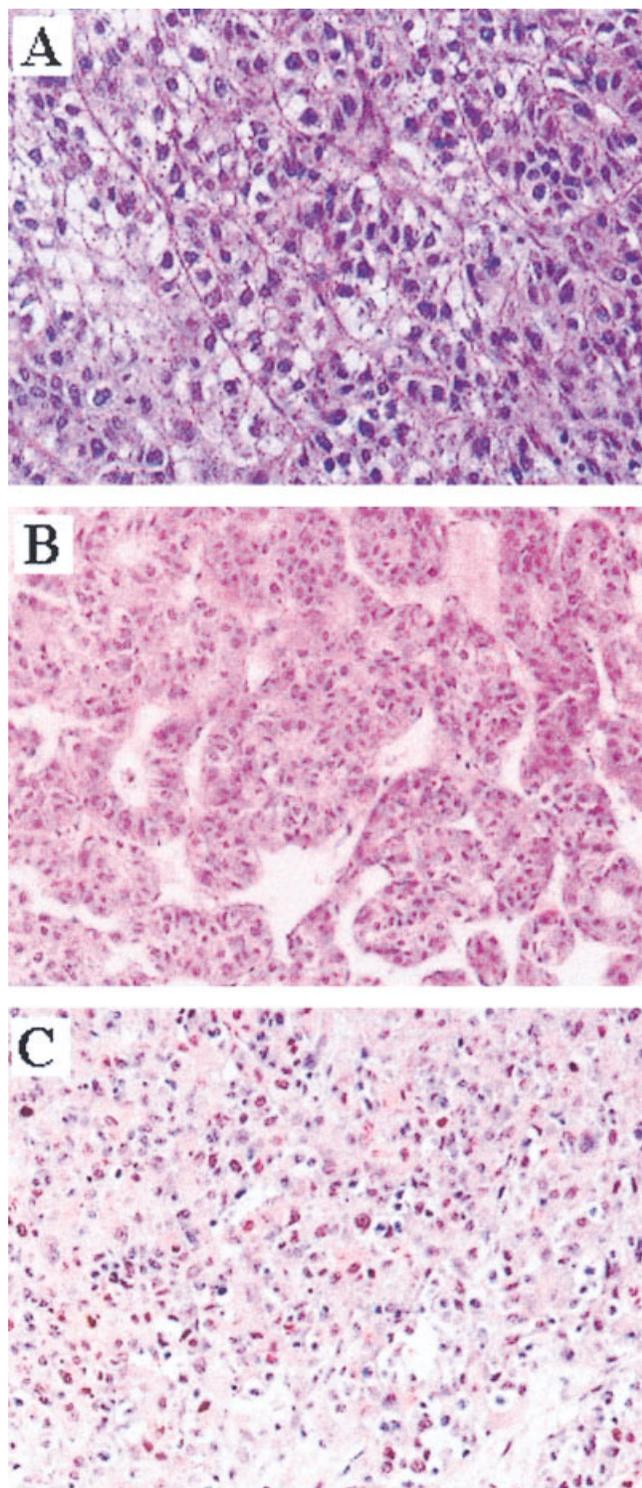
### Mutations in tumour DNA

Presence of a mutation at codon 249 was investigated by RFLP and positive samples were analysed by sequencing. Using serial dilutions of DNA from a cell line expressing Ser-249 p53 (PLC/PRF/5) in normal, lymphocyte DNA, we have shown that this method allows detection of up to 3% of Ser-249 DNA in 97% of wild-type DNA (data not shown). All analyses were repeated at least twice and 8 specimens that consistently exhibited a detectable band corresponding to unrestricted material were considered as "positive". Sequencing of the positive (uncut) RFLP fragment after amplification using nested PCR confirmed the presence of Ser-249

TABLE I—PATIENTS, HBV/HCV STATUS AND HISTOLOGICAL CHARACTERISTICS OF THE TUMOURS<sup>1</sup>

ID	Sex	Age (years)	AFP	Diagnosis	HBV status	HBsAg	Anti-HCV	p53 immuno-staining
3	M	adult	n/a	HCC, clear cell	n/a	n/a	n/a	>50%+
4	M	38	n/a	HCC, clear cell	n/a	n/a	n/a	10–50%+
6	n/a	n/a	n/a	HCC, clear cell	n/a	n/a	n/a	—
13	F	50	+	HCC, clear cell	sag+	+	—	>50%+
21	M	50	+	HCC, clear cell	core+	—	—	—
22	M	40	+	HCC, clear cell	sag+	+	—	—
23	M	25	+	HCC, clear cell	sag+	+	—	—
25	M	27	—	HCC, clear cell	core+	—	—	>50%+
26	M	45	—	HCC, clear cell	sag+	+	—	20%+
2	M	80	n/a	HCC, Edmondson grade 1	n/a	n/a	n/a	—
11	n/a	n/a	n/a	HCC, Edmondson grade 1	eag+	+	—	—
12	n/a	n/a	n/a	HCC, Edmondson grade 1	sag+	+	—	—
14	M	51	n/a	HCC, Edmondson grade 1	eag+	+	—	10–20%+
29	F	64	+	HCC, Edmondson grade 1	sag+	+	—	—
28	M	64	+	HCC, Edmondson grade 1/2	sag+	+	—	—
7	n/a	n/a	n/a	HCC, Edmondson grade 2	sag+	+	—	—
10	n/a	n/a	n/a	HCC, Edmondson grade 2	n/a	n/a	n/a	10%+
17	M	29	+	HCC, Edmondson grade 2	sag+	+	—	—
19	F	17	+	HCC, Edmondson grade 2	n/a	n/a	n/a	—
20	M	70	+	HCC, Edmondson grade 2	sag—	—	—	>50%+
5	M	adult	n/a	HCC, Edmondson grade 3	n/a	n/a	n/a	—
8	M	32	+	HCC, Edmondson grade 3	core+	—	—	20%+
16	M	64	+	HCC, Edmondson grade 3	n/a	n/a	n/a	n/a
15	M	26	+	HCC, Edmondson grade 3	sag+	+	—	—
24	M	47	+	HCC, Edmondson grade 3	sag+	+	—	>50%+
1	n/a	n/a	n/a	HCC, Edmondson grade 3	n/a	n/a	n/a	>50%+
9	M	37	—	HCC, Edmondson grade 3	sag+	+	—	—
18	M	38	+	HCC, Edmondson grade 3/4	sag+	+	—	20–50%,
27	M	50	+	HCC, Edmondson grade 4	sag+	+	—	>50%+

<sup>1</sup>n/a, not available; AFP, alphafoetoprotein, +, > = 100ng/ml; —, <100ng/ml; sag, HBV surface antigen; core, HBV core antigen; eag, HBV e antigen; For patient 4, four distinct zones of the tumors were analysed p53 immunohistochemistry, 3 of which stained from 20–50% and 1 zone which stained 10–20%.



**FIGURE 1** – Histological features of the tumours. (a) HCC with a predominant component of clear cells (HCC-CC), hematoxylin-eosin staining. (b) Hepatocellular carcinoma Edmondson grade 1, hematoxylin-eosin staining. (c) Positive 53 immunohistostaining with CM1, antibody recognising all isoforms of *TP53* (overall over 50% positive tumour cells).

(AGG to AGT) mutant DNA in all of the specimens that were positive by RFLP. Two specimens that exhibited erratic results in RFLP were further analysed by short oligonucleotide mass spec-

**TABLE II** – MUTATIONS IN EXONS 5–8 OF *TP53* IN HCC TUMOURS AND Ser249 *TP53* IN CORRESPONDING PLASMA SAMPLES<sup>1</sup>

Tumour ID	<i>TP53</i> , exon 5–7	Matched plasma <i>TP53</i> , exon 7
1	AGG→AGT at 249 (low)	–
2	Wt	–
3	AGG→AGT at 249; GAC→GAT at 259	–
4a	Wt	–
4b	Wt	–
4c	GAT→TAT at acceptor splice site, exon 8	–
4d	Wt	–
5	Wt	–
6	Wt	–
7	Wt	–
8	Wt	–
9	AGG→AGT at 249	Wt
10	AGG→AGT at 249, CAG→TAG at 192	–
11	AGG→AGT at 249	–
12	Wt	–
13	AGG→AGT at 249	AGG→AGT at 249
14	CAG→CAA at 165	Wt
15	Wt	Wt
16	AGG→AGT at 249	AGG→AGT at 249
17	Wt	Wt
18	Wt (AGG→AGT at 249 by SOMA)	AGG→AGT at 249
19	AGG→AGT at 249	AGG→AGT at 249
20	Wt	–
21	Wt	Wt
22	Wt	Wt
23	Wt	Wt
24	Wt (AGG→AGT at 249 by SOMA)	AGG→AGT at 249
25	Wt	Wt
26	CAT→TAT at 179 (AGG→AGT at 249 by SOMA)	AGG→AGT at 249
27	Wt	Wt
28	AGG→AGT at 249	Wt
29	AGG→AGT at 249	AGG→AGT at 249

<sup>1</sup>Sample number 4 had 4 zones analysed independently. Samples number 9, 10, 11, 19 and 25 were not tested for mutations in exon 5. The analyses of exons 6 and 7 were conducted for all the samples. Only samples number 4 (all zones), 8, 18, 20, 24 and 27 were screened for mutations in exon 8. In cases marked with “–”, no plasma was available for analysis.

trometry analysis (SOMA) as described by Laken *et al.*<sup>28</sup> Both of these specimens demonstrated a clear, positive SOMA signal corresponding to Ser-249 DNA. Overall, RFLP, sequencing, and SOMA analyses indicated that 10/29 specimens (34.5%) contained a Ser-249 *TP53* mutation (Table II).

To determine whether *TP53* mutations other than Ser-249 could be detected in these tumours, specimens with sufficient material available (see Table I) were analysed by direct sequencing for mutations in exons 5–8 of *TP53* (Table II). Mutations were found in patient 26 (codon 179, CAT→TAT, His→Tyr) and patient 4 (GA→TA at acceptor splice site of exon 8). Interestingly, in the latter patient, the mutation was found in only 1 of the 4 areas of the tumour specimen analysed. Patient 14 had a silent mutation at codon 165 (CAG to CAA, Gln). Two patients with Ser-249 mutations were found to have additional mutations, 1 at codon 259 (GAC→GAT, silent Asp; patient 3) and the other at codon 192 (CAG→TAG, Gln to Stop, patient 10) (Table II). No correlation was found between the presence of a mutation (Ser-249 or other) and the histological features of the tumours.

#### Mutations in plasma and correspondence with matched tumours

From 171 HCC patients (see Material and Methods), 71 (41%) were found to be positive for Ser-249 DNA by RFLP

(Kirk *et al.*, article in preparation). Biopsies in liver tissues were available and confirmed as HCC for 17 of them. Of these 17 plasma specimens, 7 (41%) were found to contain a Ser-249 mutation detectable by RFLP. Of these 17 paired plasma and tumour specimens, 12 showed concordant results as to the 249 status by RFLP, 4 of them carrying the Ser-249 mutant and 8 being wild-type at codon 249 both in the tumour and in corresponding plasma (Table II). The 5 discordant pairs included 3 pairs that were positive in plasma but negative in tumour, and 2 pairs negative in plasma and positive in tumour within the detection limit of the RFLP assays (3% of mutant DNA). To examine whether trace amounts of Ser-249 DNA may be detectable, all specimens (plasma and tumour) negative by RFLP were further analysed using SOMA to detect the possible presence of low levels of Ser-249 DNA. All 3 tumours were found to be positive by SOMA, whereas the 2 plasma specimens were negative. Overall, the concordance between results in matched tumour and plasma pairs was 88.2% (15/17 pairs; 77% if considering only Ser-249-positive tumours; Table II). In the 2 pairs that did not show concordant results, the tissue DNA was found clearly mutant and the plasma wild type.

#### DISCUSSION

Ser-249 *TP53* mutation is frequent in hepatocellular carcinoma from regions with a high prevalence of chronic HBV infection and with high levels of aflatoxin contamination of the traditional diet. A total of 297 Ser-249 *TP53* mutations have been reported in the literature and compiled in the IARC *TP53* mutation database; 196 of them (66%) have been detected in HCC. All but 7 originate from regions where aflatoxin has been reported to be a significant contaminant of the diet. The 101 Ser-249 *TP53* mutations detected in cancers other than HCC are scattered over 23 different tumour sites, with none of them showing a prevalence higher than 2.7%. Most of the available data originate from high-incidence areas of China, in particular from Jiangsu Province (Qidong County), a well-studied region with a documented high incidence of HCC. Of the 80 *TP53* mutations described in HCC from this region, 71 are Ser-249. In a recent study of liver cancer tissues obtained by surgery or biopsy in patients from Qidong County, the Ser-249 mutation was found in 65% of the cases (Szymanska *et al.*, unpublished). However, data for other areas with high exposure to aflatoxins are still limited. In particular, only 63 HCCs from Africa have been analysed. In Mozambique, 9 Ser-249 mutations were found in 16 patients (56%). In South Africa, 6 Ser-249 mutations and 1 mutation at codon 157 were detected in 32 patients (18%).<sup>6,31</sup> In Senegal, Coursaget *et al.*<sup>7</sup> detected 10 Ser-249 mutations in 15 patients (67%).

In our study, we have found a total of 15 mutations in 13 patients (45%) with well-defined HCC (2 patients had 2 distinct mutations). Ten of these mutations (35%) were Ser-249, a prevalence lower than reported in neighbouring Senegal.<sup>7</sup> This difference cannot be attributed to a lower sensitivity of mutation detection in our series since we have used a combination of extremely sensitive assays (RFLP and SOMA). A detailed analysis of the sensitivity and specificity of this methodology has been investigated and will be described elsewhere (Caboux *et al.*, article in preparation). However, it should be noted that the study by Coursaget *et al.*<sup>7</sup> was performed on surgical specimens, whereas ours is based on needle biopsies of HCC lesions. It cannot be ruled out that the small amount of material obtained by biopsy is not thoroughly representative of the whole HCC lesion. Additionally, the case series described by Coursaget *et al.*<sup>7</sup> is small (15 cases) and hospital-based. The size of the series of HCC cases analysed here is also limited (29 exploitable cases) but has been assembled in the context of a nation-wide case-control study and may therefore be more representative of HCC in the small population of The Gambia.

Histopathological examination indicated that 9/29 (30%) HCC cases contained a predominant component of clear cells (HCC-

CC). This tumour phenotype, which is due to the accumulation of glycogen and lipids partially substituting the usual cytoplasmic constituents, is generally considered less aggressive and having a better outcome than no-clear cell HCC.<sup>32</sup> Data from the literature indicate that, although up to 40% of HCC may contain some clear cells, the diffuse HCC-CC type occurs only in a minority of the cases (about 10%.<sup>32,33</sup>). Further studies are needed to determine whether the high prevalence of HCC-CC reported here is a typical feature of HCC in The Gambia.

Presence of the Ser-249 *TP53* mutation was not correlated with tumour histological subtype, stage or grade. In contrast with previous studies on HCC from China,<sup>8</sup> the presence of this mutation did not systematically correlate with p53 protein accumulation. In particular, 5 of the tumours that were negative in immunohistochemical staining with the CM1 polyclonal antibody contained the Ser-249 mutant. It is unclear why the Ser-249 mutant may accumulate in one HCC and not in the other. This observation suggests that accumulation of the Ser-249-mutant p53 protein is not an intrinsic property of this mutation but largely depends on other factors that may differ from one tumour to the other.

The analysis of plasma DNA for the presence of the Ser-249 mutation showed positive results in 7/17 (41%) HCC cases. The existence of mutant plasma DNA was not correlated with tumour stage/grade. The prevalence of Ser-249 found in this subset of HCC cases was similar to the prevalence (38%) in our larger series of 171 HCC cases (Kirk *et al.*, article in preparation) and to that found (35%) in our previous report of 53 cases,<sup>21</sup> demonstrating consistency over time. Paired comparisons between plasma and tumour specimens indicated that 7 of 9 tumours with Ser-249, and for which plasma was available, also had the Ser-249 mutation in plasma. Negative samples were also uniformly congruent and indicate that there is a good concordance (88.5% altogether) between plasma and tumour DNA findings. This concordance is of 77% between Ser-249 positive tumour samples and matched plasma. Importantly, we did not find any positive plasma that corresponded to a negative tumour, suggesting that mutation in the plasma is a good indicator of the presence of the same mutation in the liver tumour. In a study on a series of HCC patients from Qidong and Shanghai, Jackson *et al.*<sup>34</sup> have found a 55% concordance between Ser-249 mutations in tumours and in matched plasma using a highly sensitive, mass spectrometric quantitation method. A better concordance (over 85%) was found in 2 studies of *p16INK4a* methylation in HCC patients from the Hong Kong area.<sup>35,36</sup>

In conclusion, the results presented here indicate that the prevalence of the Ser-249 mutation in The Gambia, one of the countries with the highest levels of exposure to aflatoxins in the world, is lower than reported in high-incidence areas of China. It is also lower than described in the only other study on HCC from West Africa (Senegal), although the difference is difficult to evaluate since both studies are of relatively small scale. This observation raises the hypothesis that the rate of Ser-249 formation after exposure to aflatoxin may be influenced by several factors, potentially including population differences in exposure to other environmental factors, variations in the natural history of HBV infection or differences in genetic susceptibility. Moreover, our results show that plasma DNA is an excellent source of material to assess the status of codon 249 in HCC patients. This finding opens new horizons for molecular epidemiological studies of HCC in areas of the world where aflatoxin exposure is an important etiologic factor.

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