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p53 codon 249 mutation in hepatocellular carcinomas from Nigeria

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Summary

Mutations of p53 tumour suppressor gene often occur in hepatocellular carcinoma and, in particular, codon 249 hot-spot mutation is displayed by hepatocellular carcinomas occurring in hepatitis B virus-endemic areas with high dietary aflatoxin intake. This study was done to determine the frequency of p53 codon 249 mutation in hepato-cellular carcinoma in Nigerian patients with this tumour.

Tumour samples were obtained from 18 Nigerian patients (all from the Southwest of the country) with histologically confirmed hepatocellular carcinoma by autopsy (n= 14), surgical resection (n=3) and ante-mortem liver biopsy (n=1). Fourteen of them had co-existing cirrhosis. Amplification of exon 7 of p53 gene from DNA samples of hepatocellular carcinoma tissue was undertaken by nested polymerase chain reaction followed by restriction enzyme analysis. One out of the 18 tumour samples tested (5.5%) demonstrated codon 249 mutation. This study suggests that, in Nigeria, especially the south-western region, aflatoxins appear to play a limited role in hepatocarcinogenesis.

Résumé

Les mutations du gene suppression tumoral P53 se rencontrent dans le carcinome hepatocellulaire en particulier, la mutation du codon 249 est Manl keste dans les carcinomes hepatocellularies, qui souvienent dans les zones endemiques d'hepatite virale B, à forte consoumation d'aflatoxine. Cette etude a été realisee pour determiner la frequence de la mutation P53 codon 249 dans les carcinonmes hepatocelluaries chez des patients Nigerians presentant cette tumeur. Des echantillons de tumeurs ont été obterus de 18 patients Nigerians (tous du sud-west du pays) avec des carcinsomes hepatocellularies connues par histologic après autopsic (n = 14), resection chisurgicale (n = 3) et biopsie hepatique ante mortem. Quatorze de ces maladies avaient une cirrhose assouee. L'amplification de l'exon 7 du gene P53 des echantillons d'and des tissues de chaine de polymerase encastre, suivie d'analepe enzymatique restrictive. Un sur 18 echantillon testés (5.5%) a montré'une mutation du codon P249. Cette étude montre que au Nigeria, specialement dans la region du sud-ouest, l'àflatoxine joue un role limite dans l'hepatocarcinomatogenese.

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Introduction

Hepatocellular carcinoma (HCC) occurs worldwide but has a very high prevalence in countries of south-east Asia and sub-Saharan Africa. A number of aetiological factors have been associated with the development of this highly malignant tumour, prominent among which are chronic hepatitis B virus (HBV) infection [1], aflatoxin exposure [2] and chronic hepatitis C virus (HCV) infection [3]. The relative importance of these risk factors, however, varies from one geographical region to another. In high incidence areas such as Mozambique, Senegal and southern China, HBV infection and dietary aflatoxin are the major risk factors [4-7]. Point mutations of the p53 tumour suppressor gene, almost always a selective G to T transversion at the 3rd base of the codon 249, have been found to occur at a high frequency in these areas with increased risk of exposure to aflatoxin [8,9]. Nigeria, with an estimated HCC incidence of 18.6/100,000 per year, has been categorised as an intermediate-incidence area [10]. On the other hand, aflatoxin contamination of Nigerian foodstuffs, comparable to the levels observed in southern Africa, has been reported [11,12]. Also, metabolites of aflatoxin have been detected in blood samples of Nigerian patients with HCC [13]. No study has, however, been done among Nigerian HCC patients to establish the occurrence of p53 codon 249 mutation, which is considered to be reflective of the aetiological involvement of aflatoxin in HCC development [14,15]. We therefore analysed 18 HCC DNA samples from Nigeria for the presence of this mutation.

Patients and methods

Tissue samples and DNA extraction

Liver tumour specimens were obtained from 18 Nigerian patients (15 males and 3 females, with an age range of 25 to 66 years) with histologically confirmed HCC. The tumour samples were obtained by autopsies (n=14), surgical resections (n=3) and ante-mortem needle biopsy of the liver (n=1). Two of the tumours showed hepatocholangiocarcinoma and 14 of the 18 tumours had co-existing cirrhosis. Only 6 of the 18 patients were tested for serum HBsAg and all of them were positive. All the patients resided in the south-western part of Nigeria.

Deparaffinization of cut paraffin-embedded tissue sections was done by sequential changes of xylene, 100% ethanol, 70% ethanol and water as described [16]. The tumour samples were then subjected to proteinase K digestion and genomic DNA extracted by the standard phenol-chloroform method with some modifications. The finely minced tissue was suspended in 1mL of lysis solution containing NET (10mM NaCl, 10mM EDTA & 20mM Tris-HCl, pH 8), 10% SDS

and 100µg/mL Proteinase K (Boehringer Mannheim) and incubated on a rotator at 37°C for 3 days. Additional Proteinase K was added (up to a final concentration of 200µg/mL), the mixture vortexed for 2 minutes and incubated at 55°C for 2 hours. DNA was then extracted from the lysis mixture twice with equal volumes of phenol and a mixture of 24 parts of chloroform and 1 part of isomyl alcohol. Total DNA was precipitated overnight at -20°C with 2 volumes of 100% ethanol. The DNA pellet was washed with 70% alcohol, thoroughly dried and resuspended in 100µl of 10mM Tris-HCl (pH 7.5), 1mM EDTA.

PCR and Restriction Enzyme Analysis

Exon 7 of the p53 gene was amplified by nested polymerase chain reaction (nPCR) using the following oligonucleotide primers (see Ffig. 1):

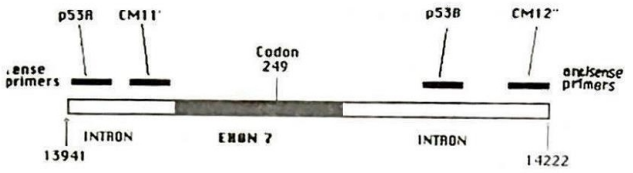

Fig. 1 - Exon 7 of the p53 gene with the adjoining introns showing the positions of the 2 pairs of primers used for nested PCR and the location of codon 249 (AGG). Initial PCR with the external primers p53A and CM12' yields a 281bp fragment which is used as a template during the 2nd PCR with the internal nested primers CM11' and p53B producing a final product of 206bp fragment length.

P53A: 5' - CTTGCCACAGGTCTCCCCAA - 3' external primer (sense)
CM 12'': 5' - GGTGGATGGGTAGTAGTATG - 3' external primer (antisense)
CM 11': 5' - CTCATCTTGGGCCTGTGTTA - 3' internal primer (sense)
P53B: 5' - AGGGGTCAGCGGCAAGCAGA - 3' internal primers (antisense)

Approximately 800ng (60ng to 2µg) template DNA was present in a 50µl reaction mixture containing 5µl of 10x thermophilic buffer (Promega Corp., Madison, WI), 1.5mM $MgCl_2$, 100µM each of dATP, dGTP, dCTP, dTTP (Promega), 15 pmols of each of the external primers and 2 units of Taq Polymerase (Promega). Positive and negative DNA controls represented by Mahlavu and Huh-7 cell lines [9] respectively were also run alongside the tumour samples. After an initial denaturation at 93°C for 5 min, the reaction mixture was subjected to 35 heat cycles consisting of 0.5min each at 94°C, 60°C and 72°C. Three microlitres of the resultant PCR products was transferred to a second tube containing the same reaction components as before but with the internal, nested primers and a further 25 heat cycles of 0.5 min each at 94°C, 58°C and 72°C carried out. This was concluded with incubation for 10 min at 72°C to ensure completion of the final extension step.

A constant 206bp fragment was produced in all the samples and this was subjected to restriction-enzyme analysis. Digestion of the exon 7 of wild-type p53 gene yields two fragments of 131bp and 75bp. In the presence of codon 249 mutation, however, a second recognition site is created and three fragments of 100bp, 75bp and 31bp are produced. The nPCR product (7.5 µl) of each of the samples was incubated in a 20µl reaction mixture containing 15 units of Hinf 1 (Boehringer Manheim, GmbH, Germany) and 2µl of buffer (H) for 2 hours at 37°C. The digestion products were then run on a 3% agarose gel and were visualised under UV light by ethidium bromide staining.

Results

Mutation at codon 249 (Fig. 2) was detected in only one of the 18 HCCs (5.5%) studied. Regrettably, the serum of the patient with this codon 249 mutation was not tested for HBsAg but he had associated cirrhosis on liver histology.

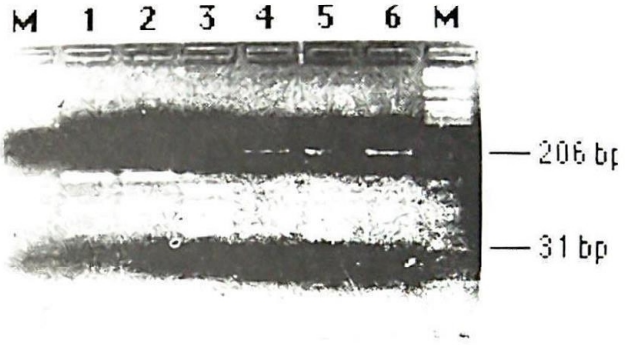

Fig. 2 - nPCR and post Hinf I digestion products.
Lanes 1 and 2 show bands at 131 and 75bp fragments resulting from Hinf I digestion of wild-type p53 exon 7 sequence. Lane 3 shows the mutated p53 gene (G to T transversion at the 3rd base of codon 249) with bands at 100, 75 and 31bp fragments. Lanes 4 to 6 show the amplified DNA fragment (206bp) containing exon 7 of the p53 gene before Hinf I digestion. M represents DNA marker or standard.

Discussion

The low rate of codon 249 mutation obtained in this study is similar to the incidence of 8% reported from Durban, South Africa [17], a country regarded as having a low risk of dietary aflatoxin intake [14]. It, however, contrasts with codon 249 mutation rates observed in Mozambique, Senegal and parts of China where high aflatoxin contamination of food has been demonstrated [8,9,18,19]. Given the reports of aflatoxin contamination of Nigerian foodstuffs [11], and the presence of detectable levels of aflatoxin in maternal and cord blood samples of Nigerians [20], codon 249 mutation rate in HCCs from the country would be expected to be higher. Perhaps, in spite of the presence of aflatoxin in Nigerian foods, the risk of aflatoxin exposure in the country is probably lower than in neighbouring West African countries as the comparative study between Nigeria and Ghana tends to suggest [20]. In a study of 22 Nigerian patients with HCC, only 22.7% were found to have serum aflatoxin levels considered to be 'pathologic' [21].

Differences in aflatoxin levels in dietary staples consumed in parts of the world may also have a role to play. Whereas most dietary aflatoxin studies in Africa and South-East Asia have consistently shown that the grains, especially

groundnuts, are the most heavily contaminated [22-24], the staple diets consumed in southern Nigeria are tubers such as yam and cassava. A report from Taiwan [25], a country where moderate aflatoxin food contamination had earlier been reported showed a low rate of codon 249 mutation (6.6%).

Taken together, these observations raise the possibilities that either codon 249 mutation requires other synergistic factors in addition to aflatoxin or that food contamination studies are not sufficient parameters for the assessment of aflatoxin exposure. On the other hand, aflatoxin does not probably play a substantial role in the development of HCC at least in southern Nigeria. However, further studies from Nigeria and other African countries with a larger sample size are needed to make firm conclusions.

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