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CLINICAL AND BIOCHEMICAL PROFILES OF TP53 CODON 249 MUTATION IN SENEGALESE PATIENTS WITH HEPATOCELLULAR CARCINOMA (HCC)

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ABSTRACT: *Hepatocellular carcinoma (HCC) is a public health issue. It is developed mainly on the bed of chronic liver diseases dominated in our contexts by carriage of chronic HBV and exposure to aflatoxin (AFB1). The aim of our study was to determine the prevalence of codons 248 and 249 mutations of TP53 gene and to study the clinical and biochemical profiles of patients with HCC in Senegal.DNA was extracted from paraffin blocks and fresh tissue from patients diagnosed with HCC. An exon 7 amplification step was followed by digestion (RLFP) with the enzymes Hae III, and Msp I which cut respectively the GG / CC fragments of codon 249 and C / CGG of codon 248.A total of 27 patients with a mean age of 43.7 were included in the study. Males were predominant with a sex ratio of 5.75. Codon 249 mutation was found in 6 patients (22.1%). 5 of them had heterozygous profile. No mutation was found on codon 248. Mutation of codon 249 was specifically found in patients with chronic HBV. Patients with codon 249 mutation were twice as likely to have an altered general condition at the time of diagnosis (RR (CI) = 2.1 (0.5-8C4)) with hyperbilirubinemia (RR = 3.4 (0.4-26.7)). Mutation was more encountered in patients with a large tumour (greater than 10 cm), localized at the right side.Our study confirms hypothetical specificity of codon 249 mutation of exon 7 of TP53 gene in hepatic carcinogenesis in patients with chronic HBV. It seems to be a good molecular marker for the prevention of this cancer in this population at risk.*

KEY WORDS: Codons 248 and 249, TP53 gene, HBV, Hepatocellular carcinoma, Senegal.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in humans and the second leading cause of cancer-related death worldwide [1]. It mainly develops in the bed of chronic liver

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conditions, such as viral hepatitis due to infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcoholic cirrhosis, fatty liver disease or exposure to toxic factors such as aflatoxin (AFB1) [2].

The spectrum of somatic variants related to hepatic carcinogenesis has been identified [3] with marked geographic variation. Mutations in TP53 (tumour suppressor protein) and CTNNB1 (cadherin-associated protein) genes are two among the most common mutations in Afro-Asian and Western countries [2].

TP53 is a gene which codes for p53 protein having a negative control over cell proliferation. Its alteration is one of the most frequent stages in cancerous transformation. The mutation of this gene totals 50% of human cancers in general and hepatic cancers in particular [4]. Several studies have shown that a mutation in the TP53 gene may play a role in hepatic carcinogenesis, particularly after exposure to aflatoxin B1 (AFB1) and to HBV. These exposures would induce a GC→AT transversion on codon 249 of its exon 7 [9,90]. The presence of this variant in hepatocellular carcinoma (HCC) is associated with a poor diagnosis [5,6].

Senegal, a Sub-Saharan developing country, is an endemic area for both HBV and AFB1 [7]. Co-exposure to these two factors has been shown to increase the risk of developing HCC by 30 [8].

The national prevalence of HBV in Senegal is very high with a chronic carriage rate of 15 to 20% which can be complicated by cirrhosis, hepatocellular insufficiency and / or HCC [9,10,11].

The only study known to have been carried out in Senegal on TP53 gene in HCC cases dates from 1993. In that study, the prevalence of codon 249 mutation was 66.7% [12].

The objective of our study was to determine the prevalence of somatic mutations in exon 7 of TP53 gene and to establish the clinical and laboratory profiles of patients with HCC in Senegal.

MATERIALS AND METHODS

Context and type of study

This was a prospective, cross-sectional, and analytical study conducted from November 2018 to September 2020 at the laboratory of biochemistry and molecular biology of the Faculty of Medicine, Pharmacy and Dentistry, Cheikh Anta Diop University in Dakar, in collaboration with the Department of surgery and anatomopathology at Idrissa Pouye Hospital (HOGIP).

Study population

Patients were recruited from the Department of surgery and anatomopathology at Idrissa Pouye Hospital (HOGIP). Selected patients were with HCC and divided into two groups:

- The first group was composed of patients with hepatectomy history and whose operative kits have been sent to the laboratory of pathology and stored in paraffin blocks.
- The second group was comprised new patients diagnosed at the department of surgery of HOGIP.

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Clinical and paraclinical data

Data were collected from patient records based on parameters such as age, sex, history, biological data (Transaminases, GGT, PAL, total and direct bilirubin, AFP, HBV serology), imaging (Echo or CT). Chronic HBV carriage was retained versus positive HBs Ag or the presence of anti-HBc antibodies for more than 6 months.

Biological specimens

We worked with paraffin blocks and fresh tissue stored at -20 ° C until DNA extraction.

Sample preparations

DNA extraction

For DNA extraction we used fresh tissue and wax tissue extraction kits from supplier Promega® (Madison, Wisconsin, USA). The protocol suggested by the manufacturer was followed.

Determination of DNA concentration and purity

The concentration of the extracted DNA was estimated by spectrophotometry at 260 nm knowing that: 1 unit of OD 260 nm = 50 µg / ml of DNA. The OD is therefore measured at 260 nm and 280 nm.

The purity of the DNA was evaluated by measuring the OD 260 nm / OD 280 nm ratio.

PCR-RLFP

Amplification of DNA by PCR

Exon 7 of the TP53 gene was amplified with the forward primers: 5'-CTT GCCACAGGTCTCCCCAA-3 'and the reverse: 5'-AGGGGTCAGCGGCAAGCAGA-3'.

During the validation of the PCR protocol, we found that the fragments from the DNA block were not sharp. To overcome this, we increased the volume of DNA by decreasing the volume of water for the preparation of the mix (Figure 1).

Hence, for the preparation of the PCR mix (50 µl) two series were carried out. One for DNA from fresh tissue (Series A) and another for DNA from paraffin blocks (Series B).

Series A: 25 µl Mix Go Taq polymerase, 3 µl DNA, 0.5 S primer, 0.5 AS primer and PCR water qs 50 µl.

Series B: 25 µl of Mix Go Taq polymerase, 20 µl of DNA, 0.2 of primer S, 0.2 of primer AS and PCR water qs 50 µl.

The PCR amplification program consisted of activation of Go Taq polymerase for 15 minutes at 95 ° C, denaturation (94 ° C, 30 s), primer hybridization (60 ° C, 30 s) and extension (72 ° C, 30

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seconds) for 50 cycles, followed by a final 5-minute extension at 72 ° C. A negative control was performed in each series of amplification to avoid the risk of contamination.

The size of the PCR products was determined by electrophoretic migration on 3% agarose gel (expected size 177 bp) with a marker of size.

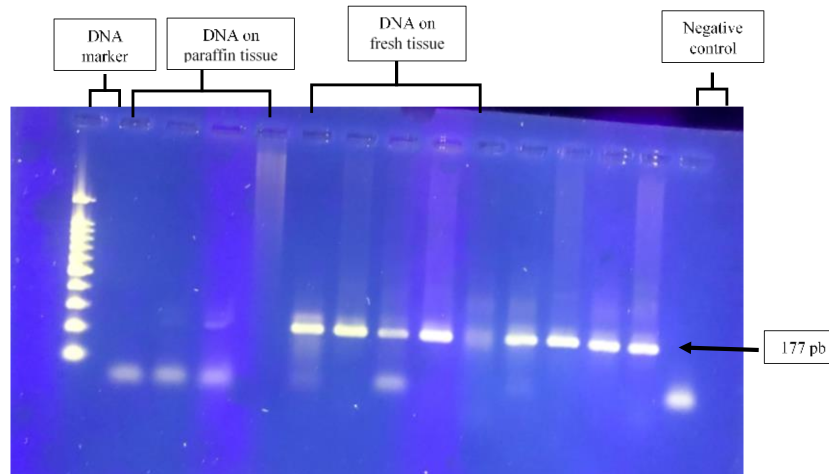

Figure 1: Electrophoretic profile of exon 7 PCR products of the TP53 gene.

Establishment of the restriction profile (RFLP)

PCR products were digested with enzymes *Hae III* (-CC / GG-) and Msp I (-C / CGG-) (New England Biolabs, UK).

The same observation was made during the digestion test with enzyme restriction on the DNA of paraffin blocks. This is how we increased their DNA volume during the preparation.

The enzymatic digestion was carried out using a 25 µl mix.

A Series: 7µl PCR product, 10µl selected enzyme Buffer (CutSmart ™ Buffer) and 3µl selected restriction enzyme and PCR water qs 25µl.

B Series: 13µl of PCR product, 10µl of selected enzyme Buffer (CutSmart ™ Buffer) and 2µl of the chosen restriction enzyme, and PCR water qs 25µl.

The mix was incubated in water bath at the restriction temperature specific for each enzyme (37°C) for more than one hour. Restriction fragments were isolated using electrophoresis on agarose gel at 3%.

Visualization of digestion products was performed under UV after staining with Diamond Dye (Promega®, Madison, Wisconsin, United States). The result is materialized by taking a photo of the gel containing coloured products.

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Under the action of the *Hae III* enzyme, the presence of a mutation at the level of codon 249 will result in an uncleaved 158 bp fragment. The same size is obtained under the action of *Msp I* for codon 248 for the mutated alleles.

Statistical analysis.

Statistical analysis was performed using SPSS v.26 software. Fisher's test was used to compare averages whereas significance level was set for values of $p < 0.05$. The bivariate analysis made it possible to search for the association between the presence of a mutation and the clinical and biochemical profiles of the patients.

RESULT

Our study population consisted of 27 patients with a predominance of male subjects (85.2). The M / F sex ratio was 5.75 with a mean age of 43.7 ± 14.25, with a predominance of subjects over 40 years (55.6%).

The study of restriction profile showed no mutation with *Msp I* enzyme for codon 248 (Figure 2A). For codon 249, mutation was found in 6 patients (22.2%). It was homozygous for one patient (3.7%) (Figure 2B).

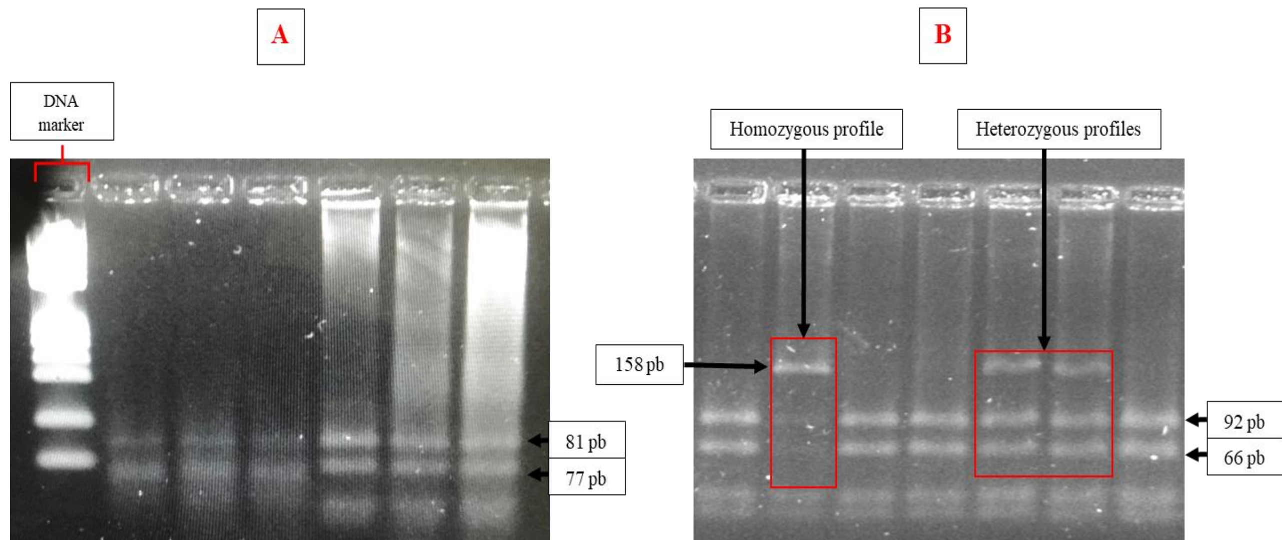

Figure 3: Restriction profile of codon 248 and 249 under the action of Msp I (2A) and *Hae* III (2B).

Comparison of averages showed no significant difference, but the mean values of all parameters were higher in codon 249 mutation carriers (Table I).

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Table I: variation of the means according to the mutation of codon 249.

	Mutation of codon 249		p
	Yes	**No**	
Age (year)	49 ± 21,8	41,4 ± 11,5	0,256
ASAT (UI/L)	189,8 ± 215,2	166,2 ± 195,9	0,801
ALAT (UI/L)	222,5 ± 360,2	224 ± 312,2	0,992
AFP (ng/ml)	385280,6 ± 859177,1	28606670,7 ± 108904293,8	0,576
BT (mg/l)	14,4 ± 6,8	14,9 ± 19,5	0,956
PAL (UI/L)	605 ± 765,1	237,6 ± 161,1	0,074
GGT (UI/L)	153,7 ± 117,8	141,8 ± 119,1	0,835

The mutation of codon 249 appears to be specific in people with chronic HBV (n = 6, 22.2%) with two patients having negative HBsAg but positive anti-HBc antibodies.

The study of the clinical and biochemical profiles of people with codon 249 mutation had shown a slightly higher risk of having an alteration in general condition (RR (CI) = 2.1 (0.5 - 8.4)), weight loss (RR (CI) = 1.4 (0.3 - 5.8)) and hepatomegaly (RR (CI) = 1.4 (0.3 - 5.5)).

Biochemically, people with mutation were subject to a high risk of developing cholestatic syndrome with increased bilirubinaemia ((RR (CI) = 3.4 (0.4 - 26.7)).

The risk of carrying the mutation did not appear to vary by age and sex, but male subjects were more likely to have mutation (n = 5, 18.5%).

Compared to the tumour, the right side location ((RR (CI) = 1.2 (0.3 - 5.3)) and the large size ((RR (CI) = 1.4 (0.3 - 6, 34)) seems to increase the risk of carrying the mutation slightly (Table II).

Table II: prevalence and association of the codon 249 mutation in the population.

		Mutation of codon 249		p	RR (IC)
		Yes (%)	**No (%)**		
Anthropometric					
Age (years)	< 40	3 (11,1)	12 (44,4)	1	0,80 (0,2-3,3)
	≥ 40	3 (11,1)	9 (33,3)		
Sex	Men	5 (18,5)	18 (66,7)	1	0,9 (0,1 – 5,6)
	Female	1 (3,7)	3 (11,1)		
Chronic HBV carriage	Yes	6 (23,1)	13 (50)	0,628	-
	Yes	0	7 (26,9)		
Clinical signs					
Hepatalgia	Yes	2 (7,7)	12 (46,2)	0,365	0,4 (0,1 – 1,9)
	No	4 (15,4)	8 (30,8)		
Jaundice	Yes	0	3 (11,5)	1	-
	No	6 (23,1)	17 (65,4)		
AEG	Yes	2 (7,2)	3 (11,5)	0,558	2,1 (0,5 – 8,4)
	No	4 (15,4)	17 (65,4)		
Weight loss	Yes	2 (7,2)	5 (19,2)	1	1,4 (0,3 – 5,8)
	No	4 (15,4)	15 (57,7)		
Hepatomegaly	Yes	3 (11,5)	8 (30,8)	1	1,4 (0,3 – 5,5)
	No	3 (11,5)	12 (46,2)		
Ascites	Yes	0	3 (13)	1	-
	No	5 (21,7)	15 (65,2)		
Characteristic of the tumour					
Lobe	Right	4 (14,8)	13 (48,1)	1	1,2 (0,3 – 5,3)
	Left	2 (7,4)	8 (29,6)		
Hight	≥ 10	4 (16,7)	10 (41,7)	1	1,4 (0,3 – 6,34)
	< 10	2 (8,3)	8 (33,3)		
Biochemical parameters					
AFP (ng/ml)	Increased	3 (15)	10 (50)	1	0,8 (0,2 – 3,8)
	Normal	2 (10)	5 (25)		
Hepatic cytolysis	Yes	4 (14,8)	13 (48,1)	1	1,2 (0,3 – 5,3)
	No	2 (7,4)	8 (29,6)		
hyperbilirubinemia	Yes	4 (15,4)	10 (38,5)	0,330	3,4 (0,4 – 26,7)
	No	1 (3,8)	11 (42,3)		
Mixed syndrome	Yes	4 (14,8)	13 (48,1)	1	1,2 (0,3 – 5,3)
	No	2 (7,4)	8 (29,6)		

RR=relative risk; IC= confidence interval

DISCUSSION

Hepatocellular carcinoma (HCC) is a public health issue in developing countries, especially in countries with high prevalence of chronic HBV and aflatoxin poisoning (AFB1). Several molecular alterations are described during this carcinogenesis, including mutations of the TP53 genes.

Our study consisted in looking for somatic mutations of codons 248 and 249 from exon 7 of TP53 gene, followed by the establishment of the clinical and biochemical profiles of patients with these mutations during HCC among the Senegalese population.

Our study population consisted of 27 patients. The mean age was 43.7 with a clear predominance of male 85.2% (n = 23, sex ratio = 5.75).

The prevalence of codon 249 mutation was 22.2% (n = 6) while no mutation was detected on codon 248. Mutation of codon 249 was heterozygous in five patients (18.5%) and homozygous in one patient (3.7%). Mutation of codon 249 was found only in chronic HBV carriers, two of whom had negative HBsAg but positive anti HBc Ab. Our results confirm the hypothesis that codon 249 mutation is specific in chronic HBV carriers [13]. A study conducted in Senegal in 1993 on 15 patients found mutation of codon 249 in 10 patients (67%). This prevalence is higher than the one found in our present study. At that time, authors concluded that this was the highest prevalence ever found in a population where the risk of aflatoxin poisoning was very high [12].

In a study carried out in Gambia on tumour tissue, Szumanska et al [14] found a prevalence of 35% (n = 10/29) of mutation in people with chronic HBV whereas Kirk et al [15] found 24.5% prevalence of 249 mutation in their study on plasma DNA in the same population. However, in the same study by Kirk et al, sequencing of exon 7 found two mutations of codon 248 which was not the case in our study.

Zulhabri's work on a Malaysian population found a higher frequency of 39% (n = 39/100) and a statistically significant difference compared to chronic HBV carriage ($p < 0.05$) [16]. In their work on plasma DNA in Gambians, Leonart et al [17] concluded that Ser249 mutation could be used as a molecular marker for early diagnosis of HCC in a population with high exposure to aflatoxin and HBV.

By comparing HCC cases and controls versus chronic HBV carriage and the presence of the codon 249 mutation, Kirk et al found a 400-fold greater risk for HCC cases.

In our study, we found that the risk related to codon 249 mutation in people with chronic HBV could not be established, whereas in Kirk's study, it was 400 times greater (OR = 399; CI 95 %: 48.6-3270) [15]. This difference could be explained by the size of the study population and the methodology used. Kirk's study was done on plasma DNA while our study was done on tissue DNA. Although several studies agree on the mutation of codon 249 (88%) in plasma DNA and in tissue DNA, a significant percentage is found in controls and cirrhotic (3.5% and 15, 5% in Kirk's study) [13,15].

We also found that patients with codon 249 mutation were subject to a greater risk of having an alteration in general condition and hepatomegaly at the time of the consultation with a respectively risk of 2.1 (0.5-8, 4) and 1.4 (0.3-5.5).

We also note that the risk of hyperbilirubinemia (RR = 3.4) was high. However, none of these patients carrying mutation had no signs of jaundice (n = 0) at the time of diagnosis

The location of the tumour at the right side increased the risk by 1.2 times, especially in patients whose tumours were larger than 10 cm (RR = 1.4). The risk does not change with advance in age, gender, and increase of AFP (RR (CI) = 0.8 (0.2 - 3.8)).

In Kirk's study, the mean age was 41.6 in carriers of codon 249 mutation and HBV. It was slightly higher in our study (49 years). This means that this age group is at risk when it comes to HCC onset. The proportion of men was higher (sex ratio = 4.6 / 1) with very high AFP rates (mean = 1000) [15].

Several studies reported that codon 249 mutation is specific in patients exposed to aflatoxin B1 [14,16]. In our study we were unable to demonstrate this exposure to aflatoxin B1.

Nevertheless, this work will continue to highlight the stigmas of exposure to aflatoxin B1 to establish a link between the presence of codon 249 mutation and exposure to aflatoxin in cases of HCC in Senegal. Finally, exon 7 sequencing will be considered to determine the exact nature of mutation in codon 249.

CONCLUSION

Our study confirms the specificity of codon 249 mutation of exon 7 of the TP53 gene in hepatic carcinogenesis in people with chronic HBV. It seems to be a good molecular marker for the prevention of this cancer in this population at risk.

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