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Metabolic Syndrome Instead of Aflatoxin-Related *TP53* R249S Mutation as a Hepatocellular Carcinoma Risk Factor

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ABSTRACT

Background: Hepatocarcinogenesis has a variety of risk factors. In Mexico City, autopsies found 14% of hepatocellular carcinomas (HCCs) without cirrhosis. **Objective:** The objective of the study was to explore if HCCs carry the *TP53* R249S mutation that has linked them to aflatoxin exposure and describe the associated risk factors. **Methods:** A retrospective review of consecutive cases of HCC was performed. Exposure to hepatotropic viruses, alcoholism, metabolic diseases, diabetes mellitus, and hypertension, as well as episodes of ascites, portal hypertension, and body mass index were retrieved. Slides were re-reviewed, macrodissected and DNA was extracted. *TP53* exon 7 was amplified, purified, and used as a template for sequencing. **Results:** In 14 years, 74 HCCs were identified in 1863 (4%) consecutive liver biopsies. No data were available in five excluded patients; the rest was submitted to exon 7 screening. Patients had a median age of 62 years, and 46 (67%) were male. Stage 4 fibrosis was observed in 46 patients (67%) and their associated risk factors were hepatitis C virus (39%, 18/46), alcoholism (20%, 9/46), hepatitis B virus (2%, 1/46), and 18 were cryptogenic. Fibrosis stage 3 or lower was observed in 23 (33%) patients without demonstrated liver disease; 8/23 had diabetes and 6/23, systemic hypertension. Steatohepatitic variants of HCC were observed in 4 and in 5, the remnant liver had steatohepatitis. A 238-bp fragment was obtained in each tumor without the expected *TP53* R249S mutation. **Conclusions:** There was no evidence of aflatoxin exposure in HCCs, with and without the known "classical" risk factors. One-third of non-cirrhotic HCCs had steatohepatitis or conditions associated to metabolic syndrome. (REV INVEST CLIN. 2020;72(5):316-22)

Key words: Hepatocellular carcinoma. Metabolic syndrome. Aflatoxin. Steatohepatitis. Diabetes mellitus. Dyslipidemia.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the second most common cause of cancer death worldwide; its risk factors are diverse and evolving, with regional trends in many societies[1]. In Mexico, mortality due to viral hepatitis, liver tumors, and cirrhosis is increasing in northern and central states[2], and HCC was the most common primary liver cancer in 2006 with a mortality rate of 4.7/100,000[3]. During the early 90s, in Mexico City, a two-fold increase in the incidence of HCC was observed among 12,556 autopsies performed over a

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25-year period[4]. Incidence of cirrhosis was 4% in that sample of middle-income patients, and 14% of HCCs were found in non-cirrhotic livers. In other studies, non-cirrhotic HCC has a frequency that ranges from 1.7% to 19%, in patients without clear associated risk factors[5-7]. A local study of 1486 cirrhotic patients identified alcoholism (39.5%), hepatitis C virus (HCV) infection (36.6%), primary biliary cholangitis (5.7%), and hepatitis B virus (HBV) infection (5%) as frequent etiologies of end-stage liver disease[8], but 154 (10.4%) of them were cryptogenic cirrhosis[8].

Tortillas (corn flour flat bread) are a staple food consumed in a daily basis in Mexico, and a recent Mexican study[9] showed that 13% contained levels of aflatoxin that exceed the allowed national quantification limits (>12 µg/kg). Aflatoxins are the carcinogenic and mutagenic mycotoxins of *Aspergillus parasiticus* and *Aspergillus flavus*. Food contamination mainly with aflatoxins type B1 (AFB1) has been associated with HCC development in Southeast Asia and sub-Saharan Africa[10], regions with a high prevalence of HBV exposure. Both are risk factors for hepatocarcinogenesis, but AFB1 induces DNA adducts and consequently promotes oxidative damage and G-T transversion in codon 249 of the *TP53* tumor suppressor gene *TP53*[10]. The R249S mutation has been found in more than 60% of HCC patients exposed to excess dietary AFB1[11-13]. In fact, two independent studies using polymerase chain reaction (PCR) and mass spectrometry analysis in tumoral and blood samples from regions with a high prevalence of HCC and AFB1 exposure had proposed *TP53* R249S mutation as a pre-diagnosis serum biomarker of HCC since 88.5% concordance between tumoral tissue and matched plasma was found[13,14]. In Guanxi, China, where >50% of HCC had the exposure, a molecular screening of all *TP53* exons showed R249S as the most frequent missense mutation in patients with and without HBV infection[15]. An earlier study analyzing 21 HCC patients from Northeastern Mexico, found 76% with AFB1 serum adducts, and of 16 amplified cases, 3 (19%) had the *TP53* R249S mutation[16]. After this single report, without a confirmatory local study, our country was considered as an intermediate risk zone for this carcinogenic pathway in the liver[16,17].

In Latin America, the genotypes H and F of hepatitis B appear to have a more benign course of disease[18], with few HCC cases associated with this infection[19,20].

Our objective was to study whether HCC in Mexican Mestizo patients carry the *TP53* R249S mutation linked to aflatoxin exposure and describe its associated risk factors in patients with and without cirrhosis.

METHODS

Data from HCC-treated patients with available liver biopsies, follow-up, and clinical information were identified in surgical pathology files from an academic medical center. Exposure to hepatotropic viruses, alcoholism, metabolic diseases, diabetes mellitus, hypertension, episodes of ascites, portal hypertension, and the body mass index were retrieved from clinical charts. Slides were re-reviewed to identify HCC and remnant hepatic tissue was reassessed for fibrosis and steatohepatitis. Macrodissection of neoplastic cells was performed; DNA was purified from paraffin-embedded tissue samples containing at least 70% of tumor cells using the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany), following manufacturer's recommendations. In all cases, the total amount of DNA obtained was greater than 300 ng. A product of 238 bp that included the exon 7 of *TP53* was amplified by PCR. The reaction was performed using 25 ng of tissue DNA, 20 µl of 2× HotStarTaq Plus Master Mix Kit (QIAGEN), and 0.25 µM forward 5´-CTTGCCACAGGTCTCCCCAA-3´ and reverse 5´-AGGGGTCAGAGGCAAGCAGA-3´ primers in a final volume of 40 µl. The PCR reaction consisted of 40 cycles in the following conditions: 98°C for 30 s, 60.8°C for 40 s, and 68°C for 40 s. PCR products were purified using QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and used as template for direct sequencing with the 3130 × l Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequence NM_000546 (GenBank) was used as a reference. The study was performed in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

RESULTS

From the years 2002 to 2015, 74 HCC patients were identified among 1863 consecutive liver biopsies (4%). No clinical data were available in five patients;

Table 1. Associated conditions and risk factors in HCC. None of the tumors exhibited the *TP53* R249S mutation of AFB1 exposure (n = 69). Data extracted from clinical records and reevaluation of tumor slides of patients with and without cirrhosis

Risk factor for HCC	Cirrhotic HCC (n = 46)	Non-cirrhotic HCC (n = 23)
Hepatitis C virus	18	0
Alcohol	9	0
Hepatitis B virus	1	0
Type 2 diabetes*	0	8
Systemic hypertension	0	6
Steatohepatitis**	0	5
Steatohepatitic HCC	0	4
Cryptogenic cirrhosis	18	0

*Six patients with type 2 diabetes mellitus (T2DM) also had systemic hypertension, two with dyslipidemia, and one T2DM, hypertension, and overweight.
**One patient had steatohepatitis in remnant liver tissue and steatohepatitic variant of HCC.
HCC: hepatocellular carcinoma.

therefore, they were excluded. Patients submitted to exon 7 screening had a median age of 62 years and 46 (67%) were male. Stage 4 fibrosis was observed in 46 patients (67%); the main risk factors for cirrhosis development were HCV infection (HCV, 39%, 18/46), alcoholism (20%, 9/46), and HBV infection (HBV 2%, 1/46), and in 18 (39%), cirrhosis was recorded as cryptogenic. Stage 3 fibrosis or lower stages were observed in 23 cases (33%) without a demonstrated chronic liver disease. Of these patients, 8/23 had type 2 diabetes mellitus (T2DM) and 6/23, systemic hypertension. Six T2DM patients also had hypertension, two had dyslipidemia, and one had T2DM, hypertension, and overweight (Table 1). Remnant liver tissue was present in 11/23 samples without cirrhosis; in five of these patients, steatohepatitis with interlaminar fibrosis stage 2 was observed. In addition, four patients had tumors with steatosis, abundant Mallory bodies, and satellitosis, one of them also with steatohepatitis in residual tissue. None of them presented the ground-glass cytoplasm nor surface/core antigen expression suggesting HBV infection, as was routinely ruled out by immunohistochemistry.

Tumor grading according to the Edmondson-Steiner and WHO modifications[21] was well differentiated (G1-G2) 31 (45%); moderate (G3) 23 (33%); and poorly differentiated (G4) 12 (17%), without differences between cirrhotic and non-cirrhotic tissue. Three (4%) fibrolamellar HCC subtypes were observed in livers without fibrosis. A 238-base pair fragment of DNA was obtained in each tumor, and its direct sequencing did not show the AGG-AGT or AGG-AGC mutation expected in codon 249 of the exon 7 of *TP53*.

DISCUSSION

AFB1 synthesized by *Aspergillus* spp. is one of the most common carcinogens in foods such as rice, cereals, fruits, vegetables, and corn, among others[17]. This toxin is chemically stable and resistant to various processes such as cooking or boiling[17]. Corn contamination with AFB1 was documented early in the 90s in Northeastern Mexico[9,16]. Recent findings of tortilla contamination with this carcinogen in Mexico City suggest extensive exposure and protean clinical expressions[9,16,22]. Its most feared complication is HCC development in regions with high prevalence of HBV infection; however, none of our cases in this study carried the *TP53* mutation linked to AFB1 exposure. Explanations for this finding could be the low prevalence of HBV as an additional risk factor in the analyzed patients (1 HCC/HBV among 69 HCC). Gain of oncogenic function of *TP53* R249S mutation depends on the microenvironmental milieu that HBV creates, leading to post-translational modifications and neoplastic cell survival advantages, as was recently described[23]. A second explanation could be that AFB1

Table 2. *TP53* R249S mutation in HCC in different countries and regions

Region	Specimen	*TP53* R249S+ve/ HCC (%)	Detection method	*R249S* mutation and serum AFB1 adducts	Year	Reference
Africa						
Southern Africa	Tumor	3/10 (33)	Sequencing	ND	1991	31
Mozambique	Tumor	8/15 (53)	RFLP	ND	1991	12
Senegal	Tumor	10/15 (67)	RFLP	ND	1993	32
Gambia	Tumor	11/18 (61)	RFLP and sequencing	ND	2004	14
Gambia	Tumor	43/56 (77)	RFLP	ND	2012	33
Nigeria	Plasma	6 (7)	RFLP and sequencing	ND	2008	34
Egypt	Tumor Serum	2/20 (10) 1/76 (1)	RFLP and sequencing	ND ND	2008	35
Asia						
Qidong, China	Tumor Plasma	11/18 (61) 2/130 (2)	RFLP SOMA	ND ND	2009	36
Guangxi, China	Tumor	18/50 (36)	Sequencing	ND	2001	37
Beijing, China	Tumor	1/116 (1)	PCR-SSCP and sequencing	ND	2013	38
Thailand	Tumor	6/25 (24)	SOMA	ND	2005	39
Thailand	Plasma	29/84 (35)	SOMA	ND	2012	40
Japan	Tumor	3/279 (9)	Sequencing	3/3	2011	41
Japan	Tumor	3/34 (9)	PCR-SSCP and sequencing	ND	1993	42
Japan	Tumor	7/140 (5)	PCR-SSCP and sequencing	ND	1992	43
Japan	Tumor	0/59 (0)	PCR and OH	ND	1993	44
Singapore	Tumor	0/44 (0)	RFLP	ND	1995	45
India	Tumor	2/21 (10)	PCR-SSCP and sequencing	ND	2000	46
Europe						
Italy and France	Tumor	0/7 (0)	Sequencing	ND	1993	47
Great Britain	Tumor	0/19 (0)	PCR-SSCP and sequencing	ND	1992	48
Germany	Tumor	0/20 (0)	Sequencing	ND	1995	49
Italy	Tumor	0/20 (0)	Sequencing	ND	1995	50
Turkey	Tumor	1/50 (2)	RFLP	ND	2010	51
America						
Brazil	Tumor	21/74 (28)	RFLP	ND	2009	52
United States	Tumor	4/37 (11)	RFLP	ND	1993	53
Hispanics in South Texas	Tumor	3 /41 (7)	RFLP	ND	2018	54
Alaska	Tumor	0/13 (0)	Sequencing	ND	1995	55
Colombia	Tumor	4/38 (11)	RFLP and sequencing	0/4	2011	56
Colombia	Tumor	1/30 (3)	RFLP	ND	2019	57
México	Tumor	3/16 (19)	Sequencing	2/3	1996	16
Mexico	Tumor	0/69 (0)	Sequencing	ND	2020	Present study

RFLP: restriction fragment length polymorphism; PCR-SSCP: polymerase chain reaction and single-strand conformation polymorphism; OH: oligonucleotide hybridization; SOMA: short oligonucleotide mass analysis; ND: not determined.

exposure became inconstant once the liver mass was present, leading to disappearance of its mutational signature through clonal and subclonal evolution of HCC[24]. Although a previous report states AFB1 exposure as a concern for HCC in Mexico[16], our sample with only one case of HBV infection is not confirmatory; instead, it should lead to consider other players in hepatocarcinogenesis[8], since hepatotropic viruses and alcoholism merely explained 40.5% of the analyzed cases (28/69).

The frequency of non-cirrhotic HCC in our study was 33%, higher than the previously observed range of 1.7-19%[5-7,25]. Adding cases of cryptogenic cirrhosis – where no known risk factor leading to cirrhosis was found – to non-cirrhotic HCC cases, the percentage of HCC without a well-documented risk factor was almost 60% in the present study. Aside of hepatotropic viruses and alcohol, new risk factors have been associated to HCC development[26] and should be analyzed in future studies in Mexico, a country where HCC prevalence in postmortem studies rose from 0.35% in 1965-1969 to 0.69% in 1985-1989[1,4].

Fibrolamellar hepatic carcinoma, the carcinoma subtype usually observed in non-cirrhotic liver[27], was identified in three patients, four HCCs were steatohepatitic[28], and the rest were conventional HCCs with trabecular and acinar patterns, none of them with the *TP53* R249S mutation. Non-tumoral liver tissue showed steatohepatitis in five patients with fibrosis Stage 3 or lower. In a country with high prevalence of obesity and diabetes as is Mexico[29], our results suggest that the metabolic syndrome could be involved in patients developing HCC without cirrhosis[30].

Although it is important to recognize the limitation of the present retrospective series lacking AFB1-albumin adducts level measurement in serum[16,17], in concordance with other studies (Table 2), our negative result reflects the wide variations of this association around the globe[31-57]. Without diminishing credibility to the previous studies also lacking adduct exploration in serum[36,38-40], two studies done in Asia showed lower frequencies of *TP53* R249S in that region[37,41], and there is the pioneering study of Mexican patients done abroad in a collection of only 16 liver tumors and plasma[16]. In our setting, where end-stage liver disease screening is exhaustive, steatohepatitis and conditions associated to metabolic syndrome warrant the reassessment of clinical records and liver tissue beyond the well-known "classical" risk factors for hepatocarcinogenesis. The silent natural history of liver lipotoxicity is starting to be unveiled in Mexico[58], and more and larger studies on chronic liver damage, with in-depth clinical and molecular analyses, are required to elucidate further our interesting findings.

In the absence of HBV infection, no evidence of aflatoxin involvement in hepatocarcinogenesis was revealed in this retrospective study. More than half of HCC lacked a known risk factor for end-stage liver disease. In addition to the traditional risk factors, steatohepatitis or conditions associated with metabolic syndrome were frequent in 69/1863 (4%) HCC obtained from consecutive liver biopsies.

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