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p53 IMMUNOSTAINING PATTERN IN BRAZILIAN PATIENTS WITH HEPATOCELLULAR CARCINOMA

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SUMMARY

Hepatocellular carcinoma (HCC) is an important type of cancer etiologically related to some viruses, chemical carcinogens and other host or environmental factors associated to chronic liver injury in humans. The tumor suppressor gene p53 is mutated in highly variable levels (0-52%) of HCC in different countries. **OBJECTIVE.** The objective of the present study was to compare the frequency of aberrant immunohistochemical expression of p53 in HCC occurring in cirrhotic or in non-cirrhotic patients as well as in liver cell dysplasia and in adenomatous hyperplasia. We studied 84 patients with HCC or cirrhosis. **RESULTS.** We detected p53 altered immuno-expression in 58.3% of patients in Grade III-IV contrasting to 22.2% of patients in Grade I-II ($p = 0.02$). Nontumorous areas either in the vicinity of HCC or in the 30 purely cirrhotic cases showed no nuclear p53 altered expression, even in foci of dysplasia or adenomatous hyperplasia. No significant difference was found among cases related to HBV, HCV or alcohol. **CONCLUSION.** The high frequency of p53 immunoexpression in this population is closer to those reported in China and Africa, demanding further studies to explain the differences with European and North American reports.

KEYWORDS: Liver cancer; p53; Cell cycle; Apoptosis; Hepatitis B; Hepatitis C; Immunohistochemistry.

INTRODUCTION

Hepatocellular Carcinoma (HCC) is the most frequent type of primary liver cancer and one of the commonest cancers worldwide, and, in some countries in Asia and Africa, a leading cause of deaths⁵⁰. Due to its heterogeneous geographical distribution, HCC has been instrumental in the identification of major risk factors. HCC is etiologically linked to viruses (Hepatitis B and Hepatitis C), chemical carcinogens (aflatoxins) and other host or environmental factors that cause chronic liver injury^{8,9,45}. These factors trigger a sequence of events leading to malignant transformation of liver parenchymal cells. Indeed, the previously normal liver (quiescent cells) after chronic exposure to different risk factors, in advanced stages of cirrhosis with repetitive bouts of cell death, regeneration and proliferation acquire preneoplastic features. The oxidative stress and the inflammatory cytokines liberation triggered by this process may cause serious genetic lesions that govern cell proliferation (clonal distribution) and the genesis of the tumour.

Since 1991, genetic studies have shown high prevalence of point mutations in p53 in southern Africa and in the region of Qidong, China, areas with high prevalence of viral infections as well as high concentration of aflatoxins in dietary intake^{23,30}. The prevalence is much lower in other parts of the world.

Cancer cells are characterized by unrestrained growth which may be due to an imbalance between cell proliferation and death. Recently, several studies have shown that various types of alterations in cell cycle regulators are found in many kinds of cancer. p21^{Waf1/Cip1} (p21) and p27^{Kip1} (p27) are part of this cell cycle regulators³¹. They belong to a family of genes, which negatively regulate the cell cycle, therefore, inhibiting the cell proliferation. The cell cycle is positively controlled by complex of cyclins and cyclin-dependent kinases (CDK)^{11,50}. The main substrate of cyclin and CDK is the retinoblastoma gene product (Rb). Phosphorylation of Rb by cyclin/CDK complex allows progression of the cell cycle. p21 and p27 by binding to cyclin/CDK abrogate their activity^{40,41,43}. Consequently, Rb is not phosphorylated and cell cycle progression is arrested. Hence, p21 and p27 are small proteins that have the ability to halt the cell cycle proliferation by inhibiting the cyclin/CDK complex, acting as tumour growth suppressors. Loss or decreased expressions of p21 and/or p27 may facilitate the proliferation of cancer cells.

It is accepted that cancer proceeds through the accumulation of mutations in genes that govern cell proliferation and death. In recent years, the most commonly observed genetic alterations in HCC, as well as in many other tumours affecting humans, have been reported to be the mutations of p53 gene⁵⁰.

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The anti-oncogene p53 is located on chromosome 17p and contains 11 exon interrupted by 10 introns, down-regulates cell proliferation by coding for a nuclear phosphoprotein which binds to specific DNA sequences, arresting cell growth at late G1 phase of the cell cycle. This protein has a half-life of about 20 minutes and so is not regularly detectable by immunohistochemistry (IHC).

Most p53 mutations are missense and produce a faulty protein called mp53, functionally inactive but chemically stable. So, this gene has the features of a tumour suppressor gene in its wild type form and can be a dominant oncogene in its mutated form. Since its discovery two decades ago, the diverse role of p53 provided an explanation why mutations in this gene drive a normal cell towards cancer³¹.

The most common techniques used for p53 mutation detection include PCR based assays like Single Strand Conformational Polymorphism (SSCP), Denaturing Gradient Gel Electrophoresis (DGGE)²⁶ and DNA Sequencing. Recently, another method based on yeast functional assay was developed to detect p53 mutations. However, most genetic alterations are point mutations between codons 120 and 290 of the 393 amino-acid residues leading to mp53 and these can be detectable in the liver cell nucleus by IHC.

The p53 gene is mutated in about 27% of HCCs worldwide¹¹. All reported p53 mutations in HCC are somatic; therefore, germ line mutations of p53 appear not to predispose to HCC. Recently, the p53

overexpression was associated with poor prognosis in patients with HCC²⁸.

Both the frequency and the type of p53 mutations are different depending on geographic location and suspected etiology of these tumors. The frequency of all p53 mutations varies between 0 to 20% in North America and Europe and 42 to 52% in China. Although these mutations may be present independent of HBV or HCV infection, some studies describes higher indexes with aflatoxin exposure, either alone or associated to viral infections⁵⁰ (Table 1).

The mutational spectrum of p53 gene associated to a given tumor is important in the etiology of cancer and it can be exploited to therapeutic and prognostic aims. Cancers presenting p53 mutations tend to be more aggressive and chemotherapy resistant than cancer of similar tissues expressing wild type p53.

Since most missense mutations of p53 produce stable protein, high levels of agreement are shown between IHC and genetic studies. However, a mutant case could result negative at IHC due to excessive fixation or when the genetic alteration is a deletion or a non-sense mutation, with no production of any p53 protein.

On the opposite, IHC may result positive in the absence of mutation, for instance when there is an abnormal accumulation of non-mutated p53 protein, most probably related to post-transcriptional phenomena.

Table 1
Frequency and type of alteration and the methodology applied worldwide *

Geographical area	Alteration rate	Affected codon	Method	Correlations	Ref.
United States	6/30 (20%)	exons 4-8	IHC, SSCP	HCV	28
Alaska	0/13	—	PCR sequence	—	13
Japan	123/439 (28%)	exons 5-8	SSCP, PCR sequence	17p and 13q LOHs, viral hepatitis, late tumor stage, large tumor gradelarge tumor size, poor differentiation	21
Thailand	2/15 (13%)	249,254	PCR sequence	—	22
China (Qidong/ Shanghai)	73/139 (52%)	Mostly 249	PCR sequence	HBV, AFB1, 17p LOH, 4q LOH	20, 47
China (Hong Kong)	6/21 (29%)	exons 5-9 (249:13%)	PCR sequence	Progression	37
Taiwan	66/211 (31%)	exons 5-8 (249:13%)	PCR sequence, SSCP, IHC	Late tumor stage, HBV/AFB1/249	15
Germany	10/55 (18%)	exons 5-8	SSCP, PCR, cloning	—	31
Britain	59/231 (25%)	exons 5-8	SSCP, DGGE	Viral hepatitis, poor prognosis haemochromatosis (220)	10
France	9/58 (15%)	—	IHC	Viral hepatitis, tumor size, invasion	32
Spain	0/129	—	SSCP, IHC	—	5
Mozambique	5/10 (50%), 249	249	PCR sequence	17P LOH, AFB1	6
Senegal	10/15 (67%), 249	249	PCR sequence	AFB1	12
Australia	0/15	—	PCR sequence	—	50

* Modified from BUENDIA, 2000⁷.

Since major functions of p53 in the cell cycle and cell transformation are performed directly by its protein, IHC may serve as a very useful tool for the study of p53 alterations in carcinogenesis⁵⁷.

The development of HCC appears to be preceded by liver cell dysplasia, a condition subject to controversy and confusion. First, ANTHONY *et al.*³ suggested the preneoplastic capacity of liver cell dysplasia and then several studies have stressed the relation with carcinoma. Recently, LIBBRECHT *et al.*³⁸ investigated the predictive value of small and large cell dysplasia, revealing large cell dysplasia as an identifier of patients at risk for developing HCC.

In Japan, the presence of a large regenerative nodule within a cirrhotic liver, referred to as a macroregenerative nodule or adenomatous hyperplasia is thought to play a role in the pathogenesis of hepatocellular carcinoma⁴. In hepatocarcinogenesis of the cirrhotic liver, several Japanese authors suggest that the adenomatous hyperplasia might be the first step in the development of HCC, going through phases of dysplastic nodule and small HCC in a multistep fashion^{4,34,48}.

The objective of the present study was to compare the frequency of abnormal immunohistochemical expression of p53 phosphoprotein in HCC occurring in cirrhotic versus non-cirrhotic patients as well as in large liver cell dysplasia (large cell dysplasia) and in adenomatous hyperplasia.

CASUISTIC AND METHODS

We studied 84 patients with hepatocellular carcinoma or cirrhosis. 54 patients were cases of hepatocellular carcinoma; 39 of them were cirrhotic and 15 non-cirrhotic patients. The other 30 patients were cirrhotic patients without hepatocellular carcinoma. Records were retrieved from the Hepatology Branch, Department of Gastroenterology or Division of Pathology of Hospital das Clínicas, São Paulo University School of Medicine.

Immunohistochemical data analysis: All reactions were performed at the Immunohistochemistry Laboratory, Adolfo Lutz Institute, the Public Health Laboratory, São Paulo, Brazil.

The following primary monoclonal antibodies used were DO-7 (Dako, USA), 1801 (Bio-gennex, USA) and DO-1 (Santa Cruz, USA). The epitopes recognized by the antibodies are located between the N-terminal amino acids 1-45 (DO-7), 46-55 (1801) and 37-45 (DO-1) of the human p53 protein. Four-micrometer-thick histopathological sections were prepared from formalin-fixed paraffin-embedded tru-cut needle liver biopsies. Antigen retrieval was achieved with pressure cooker for 3 minutes. The specimens were incubated overnight with the primary antibody at 4 °C. Amplification was achieved by streptavidin-biotin-peroxidase (Dako Duet, USA) and, in 4 cases with cytoplasmic p53 staining where samples were still available, a new reaction amplified with polymer-enzyme Envision-Alkaline Phosphatase System (Dako, USA) was performed, thus assuring that reactivity was not due to endogenous peroxidase or biotin (Fig. 1). The reaction products of peroxidase were visualized by amplification with avidin-biotin-peroxidase complex (Vector Laboratories, USA) and developed with H₂O₂ and diaminobenzidine (Sigma Corp., USA).

One observer (VAFA) blinded to the clinical outcome of patients performed assessment of staining²¹.

Chi square test was used for statistical analysis. All *p* values reported are for a two-sided test, and the level of significance was set at 0.05.

RESULTS

In the present study, antibodies DO-7 and DO-1 yielded quite similar results, with sharp nuclear staining in many neoplastic hepatocytes and occasionally also in the cytoplasm of some tumor cells. Antibody 1801 was shown less sensitive with fewer cases positive, showing rather faible nuclear staining.

In our sample, considering all the 54 patients with HCC, p53 immuno-expression was found in 19 patients (35.2%) (Fig. 1). Out of 39 cirrhotic patients with HCC, nuclear p53 expression was detected in 13 (33.3%). The comparison of p53 immuno-expression in 6 of 27 well and moderately differentiated HCC (grades I or II)^{19,35,39} versus the 7 of 12 poorly differentiated (grades III or IV)^{18,30,34} a statistically significant difference in the results obtained (Fig. 2). Nontumorous areas either in the vicinity of HCC or in the 30 purely cirrhotic cases showed no p53 expression, even in foci of large cell dysplasia or adenomatous hyperplasia. The reproducibility of this staining pattern in the 4 cases where samples were still available for reaction with the recently introduced Envision System-Alkaline Phosphatase demonstrate this is a genuine pattern. These data shows a very high immuno-expression of abnormal p53 in Brazilian patients mostly in those with high-grade advanced tumors suggesting that p53 alteration occurs late in the carcinogenesis. When we considered HBV, HCV or alcohol in the cases studied, no significant difference was detected. In the noncirrhotic HCC group the p53 expression was detected in 6 in 15 cases (40%), slightly higher than in those occurring in cirrhotic patients.

DISCUSSION

Abnormal immunohistochemical expression of p53 was detected in 35.2% in the present Brazilian group of HCC patients, in a remarkable similarity to the findings from PAIVA *et al.*, reporting p53 immunoexpression in 5/15 HCC cases also from São Paulo, Brazil⁵¹. Several studies have indicated a relation between the p53 gene alteration and the development of HCC⁴¹. This alteration occurs in the HCC of different causes, including HBV and HCV infection, aflatoxin intake, hemochromatosis, and alfa1-antitrypsin^{1,17,30,56}. This alteration in p53 is linked to cellular events which are accompanied by increased expression of several growth factors that enhance the survival of carcinogen-activated cells by suppressing apoptosis and increasing elements entering the cell cycle^{17,45}. Trying to take this correlation in order to improve the diagnosis and the prognosis knowledge of this tumor, we studied the p53 immuno-expression using a immunohistochemical method with monoclonal antibodies to different epitopes of N-terminal region of p53 DO-7, 1801 and DO-1.

We could not find significant differences in the p53 expression in cirrhotic *versus* noncirrhotic patients with HCC contrasting to results of high-throughput gene analysis using cDNA microarrays study which supported the idea of different genetic alterations in HCC with or without cirrhosis⁵³.

Higher detection of p53 alteration in advanced grade tumor (GRADE III-IV) and absence of that alteration in dysplastic and adenomatous hyperplastic areas in the vicinity of the tumor are in agreement with a

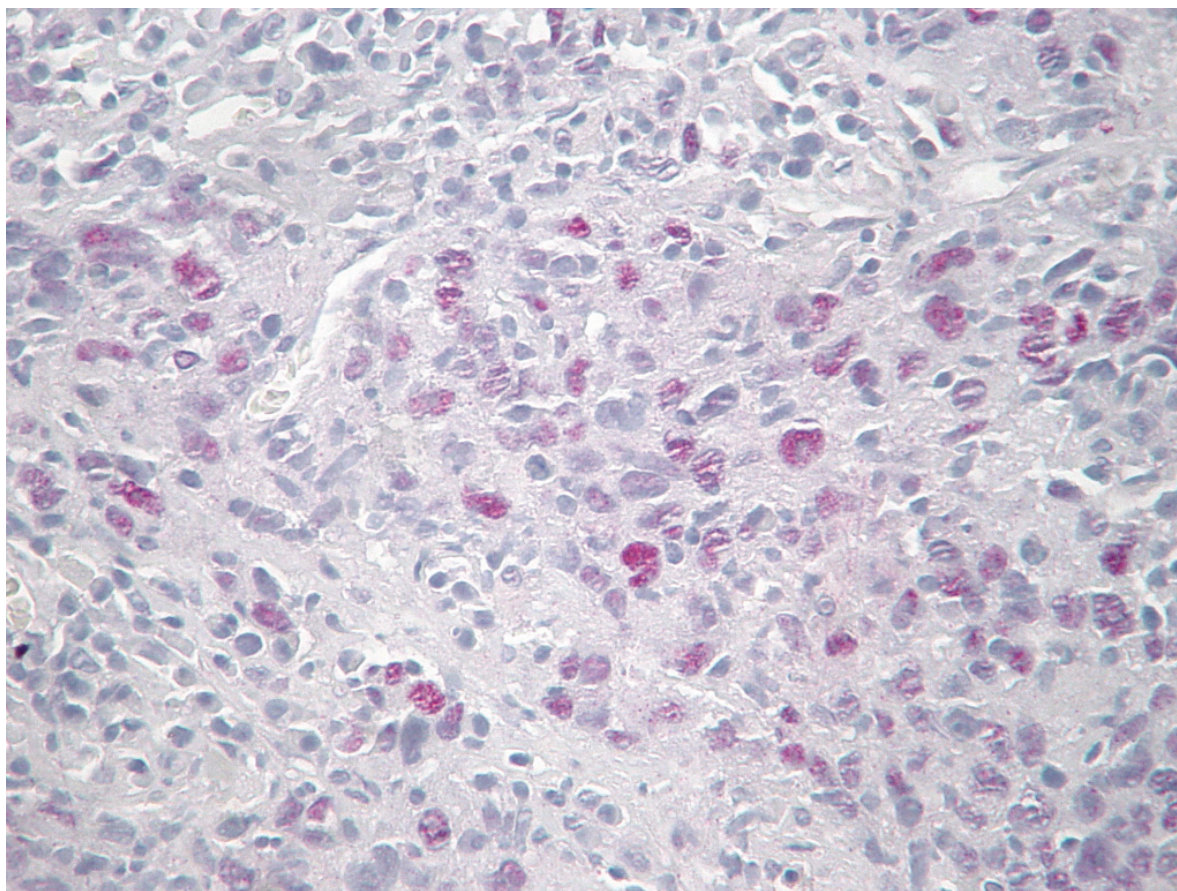


Fig. 1 - Immuno-expression of p53 is abundant in nuclei, whereas cytoplasmic immunostaining is found in occasional neoplastic cells (DO-7, Envision Alk. Phosphatase, x 200).

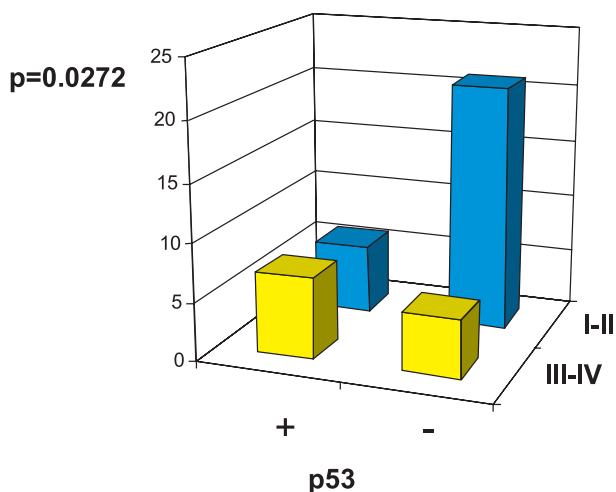


Fig. 2 - p53 immuno-expression versus histological grade.

research which shows p53 gene alteration of 15% and 52% in moderately and poorly differentiated HCCs respectively and not at all in well differentiated HCC. Since in the preferential pathway for liver cell carcinogenesis, early/small hepatocellular carcinomas are usually well-

differentiated (grades I and II) whereas advanced/large carcinomas are preferentially grades III-IV, this finding further demonstrates that p53 alterations are late event in hepatocarcinogenesis. Another study that examined p53 gene by PCR-RFLP also corroborates to this conclusion, showing a p53 mutation rate of 10%, 55.2% and 80.8% in the well-, moderately- and poorly-differentiated HCC respectively³². In contrast, it was demonstrated that in animals the p53 gene mutations is present in initial hepatocarcinogenesis²², and HUANG *et al.* showed that in humans a serine substitution at codon 249 of the p53 gene might serve as a new early diagnostic marker for HCC²⁹.

Moreover, it was showed in a Japanese study that the spectrum of p53 mutation did not differ among HCCs in relation to the type of hepatitis virus infection, sex, age, and background liver disease of patients, tumor size or presence of metastasis, however incidence and site were significantly associated with the degree of differentiation of cancer cells. Using Single Strand Conformation Polymorphism analysis, it was found 54% of p53 mutation in poorly differentiated HCC while the mutation was less frequent (21%) in well or moderately differentiated HCC⁴⁶.

p53 gene mutation is suggested to occur independently of the type of infection or status of preexisting liver disease, as well showed in our research where HBV, HCV or alcohol was not a factor of difference in the mp53 expression⁴⁶. However, in a recent study, ALTAF found p53

immunoexpression in 52% of cases and 53% of them were having anti hepatitis B surface antigen positivity, detected in their serum and 23% were having hepatitis C antibodies positive². On the other hand, DING *et al.* found only 20 p53 point mutation at exon 7 in 239 HCC samples examined¹⁶.

In a study using immunohistochemical detection for mutant p53 protein with monoclonal antibody Pab1801, TERAMOTO *et al.* found p53 overexpression in 22 tumours (31%) but in none of the non-tumorous liver specimens⁵⁴. Overexpression of p53 was more frequent in poorly differentiated tumours ($p = 0.01$), in tumours > 5 cm ($p = 0.05$), and in those presenting giant cells ($p = 0.03$). No significant correlation with hepatitis B or C status, background liver disease was detected, suggesting that p53 mutation in HCC was a late event in HCC⁵⁴. The present study, along with other papers further corroborate this hypothesis^{2,60}.

In a study that has assessed p53 expression by immunoluminometric assay on liver samples from 40 patients with biopsy-proven HCV-related chronic hepatitis, was found an overexpression of p53 in 17.5% of them and this p53 positive group had a daily ethanol intake significantly higher in respect to that of the p53 negative group ($p < 0.05$)¹⁸. Another study found that tumors associated with alcoholism had more frequent alterations in the p53 pathway than those caused by HCV infection²⁰. In our study, a different factor related to carcinogenesis of HCC is also evident, since no difference was found among patients with HBV, HCV or alcoholic influence.

Regarding the less commonly reported finding of cytoplasmic p53 immunoexpression, the reproducibility of results in cases with enough sample in the block for the repetition of the reaction with the new polymer-enzyme Envision method reinforces the hypothesis that this may be an odd, but genuine localization of p53, as previously suggested by ZHAO *et al.*⁵⁹ and by ZERBINI *et al.*⁵⁸. In a recent experiment OH *et al.* showed that bile duct ligation shifts p53 immunohistochemical expression from nuclei to cytoplasm and advocate that such shifting could play an important role in induction of apoptosis⁴⁷.

CONCLUSION

In this study we tried to correlate the immunohistochemical expression of altered p53 (mp53) to the degree of alterations of hepatic cells.

We found a frequency of mp53 expression in HCC of 35.2%, which is intermediate between data from USA (6-18%) and Africa (40-50%) where this alteration would be related to aflatoxin. Moreover, it was not found a significant difference between cirrhotics versus non-cirrhotics, as well as among HBV and HCV and alcoholic suggesting another triggering factor for such alteration in Brazil. The higher frequency of aberrant immuno-expression of p53 in less differentiated HCCs further demonstrates that p53 alterations are late phenomena in liver carcinogenesis.

Future studies should be aimed at mapping hot spot areas of p53 mutation as well as at assessing the possible role of other etiological agents, including toxics, such as aflatoxins and organic contaminants in HCC in Brazil.

RESUMO

Expressão imunohistoquímica do p53 carcinoma hepatocelular de pacientes brasileiros

O carcinoma hepatocelular (CHC) é um importante tipo de câncer relacionado etiologicamente a alguns vírus, carcinógenos químicos e outros fatores ambientais que causam danos crônicos ao fígado em humanos. A frequência de mutação do gene p53 em CHC é altamente heterogênea (0-52%) nos diversos países. OBJETIVO. O objetivo deste estudo foi determinar, imuno-histologicamente, a frequência da expressão anômala de p53 em CHCs em pacientes cirróticos versus não-cirróticos, bem como em displasia hepática e hiperplasia adenomatosa. Para isso, foram estudados 84 pacientes com carcinoma hepatocelular ou cirrose. RESULTADOS. Foram detectadas expressões do p53 alterado em 58,3% dos pacientes com CHC graus III-IV, contrastando com os 22,2% dos pacientes com CHC graus I-II ($p = 0,02$). Áreas não tumorais, tanto nas proximidades do CHC como nos 30 casos de cirrose não mostraram expressão nuclear alterada do p53, mesmo nas displasias ou hiperplasias adenomatosas. Quando se considerou HBV, HCV ou alcoolismo nos casos estudados, não se encontrou diferença significativa. CONCLUSÃO. A elevada frequência de imuno-expressão de p53 nesta população é próxima à relatada na China e África, tornando necessárias outras pesquisas para explicar as diferenças com os CHC estudados na Europa e na América do Norte.

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