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Analysis of p53 Protein Expression in Hepatocellular Carcinoma

Florin Graur[1,2], Luminita Furcea[1,2], Emil Mois[1,2], Andrei Biliuta[1], Aliz-Timea Rozs[2], Vasile Negrean[2,3], Nadim Al Hajjar[1,2]

1) Prof. Dr. Octavian Fodor Regional Institute of Gastroenterology and Hepatology;
2) Iuliu Haţieganu University of Medicine and Pharmacy;
3) Universitary Hospital CF, Cluj-Napoca, Romania

Address for correspondence:
Vasile Negrean, MD, PhD
Iuliu Haţieganu University of Medicine and Pharmacy,
Universitary Hospital CF
Cluj-Napoca, Romania
Vasile.Negrean@umfcluj.ro

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ABSTRACT

Background & Aims: Hepatocellular carcinoma (HCC) has a growing incidence and studies regarding the risk factors or pathogenesis for this type of carcinoma benefit special interest. This study evaluates the correlations between p53 protein expression and clinical and laboratory factors in patients withHCC.
Methods: The study group included 76 patients diagnosed with HCC, either by biopsy or after surgical resection (with curative intent). Immunohistochemistry for p53 protein assessment was performed in all patients. Correlations between the protein 53 expression and age, tumour size, viral infection, liver cirrhosis were performed using the chi-square test (Pearson's chi-square) together with the contingency coefficient Kendall's coefficient in the tau-b form.
Results: In the study group, 51 patients were male (67%) and 25 female (33%). Cirrhosis due to hepatitis virus B or C infection (in a proportion of 63% of the study group) was not significantly associated with the presence of HCC. Altered expression of p53 protein was observed in 69 patients (91%). The relationship between p53 protein expression and patient sex (p=0.067), age (p=0.531), tumour size (p=0.270), presence of hepatitis B and C viral infections (p=0.7), and of liver cirrhosis (p=0.511) was not statistically significant.
Conclusion: The p53 protein expression was not significantly associated with the demographic characteristics of the patients, tumour size, presence of viral B and C infections or liver cirrhosis.

Key words: hepatocellular carcinoma – p53 protein expression – HBV infection – HCV infection – cirrhosis

Abbreviations: HBV: hepatitis B virus; HCV: hepatitis C virus; HCC: hepatocellular carcinoma; TP53: tumour protein p53; MDR: multi-drug resistance gene.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the cancers with a continuous rising incidence and currently ranks the sixth among cancers in the world [1]. Risk factors for HCC have been largely described and have a direct impact on disease, making HCC a complex disease with a poor prognosis [2].

The HCC incidence varies from 2 to 30 cases/100,000 inhabitants/year [3]. In Europe and the United States of America, the incidence of HCC reaches about 45% [4], with etiological factors in varying proportions [5]. The etiological factors involved in the pathogenesis of HCC [6] can be potentially reversible factors: on one hand, lifestyle related - alcohol abuse, smoking, obesity, diabetes, and on the other hand environmental factors: viral infections (hepatitis B virus HBV, and hepatitis C virus HCV), contaminated food (aflatoxin B1) and industrial chemical agents. Genetic diseases involved in the etiology of HCC include hereditary hemochromatosis and alpha1-antitrypsin deficiency [6].

The pathogenesis is a multistage process, which varies depending on the etiology. Hepatocarcinogenesis is due to both phenotypic changes and genetic alterations. There are three essential genetic changes that occur in HCC pathogenesis: genetic amplification (due to activation of proto-oncogenes); deletion or mutation of genes (due to inactivation of proto-oncogenes) and reactivation of telomerase function. Of the many proto-oncogenes implied in HCC pathogenesis some have been more intensively studied such as tumour protein p53 (TP53), c-myc and c-fos; on the other hand, researchers have found increased levels of P-glycoprotein in the serum of

some patients with HCC in Taiwan, a change that indicates the alteration in *MDR* gene [7].

The most common gene involved in carcinogenesis is the *TP53* suppressor gene. Except for pathological situations, the p53 protein level is low. In 50% of human cancers, *TP53* suppressor gene is inactivated, and the signaling pathways leading to cell cycle arrest and apoptosis are defective [8]. The most frequent mutations in the *TP53* gene are: crossing G: C to T: A transversion at the codon 249 and C: T to A: T and C: G to T: A at the codon 250 [9, 10]. In some studies a correlation has been observed between *TP53* gene expression and survival of patients with HCC. Thus, patients with mutant *TP53* unregulated expression show a shorter survival rate than patients with wild *TP53*. The R249S mutation is most frequently observed in patients with HCC [11, 12].

TP53 mutations frequency and the spectrum of these mutations varies by geographical area, and reflect differences in etiology and susceptibility of the host organism [13]. Studies of the *TP53* gene showed that the most frequently encountered gene alteration is found in the advanced stages of the disease. Expression of p53 protein is associated with the proliferative marker Ki-67, having a significance in the advanced stages of the disease. The high levels of these two proteins may indicate the presence of tumour metastasis and portal invasion. The patients who have a high expression of the p53 protein, most often have cirrhosis and a large size tumour [14, 15].

In the present study we analyzed the expression levels of p53 protein by immunohistochemistry in patients with proved HCC and the correlation between the expression of p53 protein and the clinical and laboratory data.

METHODS

Selection of patients and data collection

A descriptive, observational, longitudinal retrospective study was performed. The study group included 76 patients diagnosed with HCC confirmed by histopathology, hospitalized between 2005 and 2010 in the Surgical Department of our Institute. A curative resection was performed in all patients using open surgery.

The following parameters were evaluated: patient distribution by age and sex, number, location and size of the tumours, histopathology of the tumours, associated pathology (HBV or HCV infection, liver cirrhosis), the tumour resection margins, staging and results of the p53 protein immunotesting. Data were collected from the pathology database of the Institute and the immunohistochemical analysis was performed for selected patients.

The informed consent for the study was given by all patients.

Immunohistochemical analysis

The DAKO PEN (Monoclonal Mouse Anti-Human p53 Protein - Clone DO-7 - Code-Nr. M 7001) from Dako, Denmark A/S was used for p53 immunohistochemistry. Monoclonal antibodies against p53 were used to mark the slices for p53 immunohistochemistry [16]. The immunohistochemistry results were expressed in percentages, and according to the international literature, the enzymatic activity was classified into four categories: 1 - absence of enzymatic activity, 2 - activity below 30%, 3 - activity between 30% and 59%, and 4 - activity over 60%. This was the classification we used in this study.

Statistical analysis

Statistical analysis was performed using Data Analysis from Excel and SPSS 19. In order to assess the relationship between the p53 protein and the aspects considered, different types of tests were applied, in accordance with the type of variables used. When nominal data was considered, the chi-square test (Pearson's chi-square) together with the contingency coefficient was applied. The latter evaluates the intensity of the relationship in case this exists: (0;0.3) - low intensity, (0.3; 0.7) - medium intensity and (0.7; 1) - high intensity. In the case of ordinal data, the Kendall's coefficient in the tau-b form was applied, as this type of data was expressed through several groups. In case any relationship exists, the coefficient also shows the direction. Significance was considered at the 5% level.

RESULTS

The risk factors for developing HCC in our patients and the occurrence of genetic modifications in correlation with the risk factors were analyzed.

Analysis of HCC occurrence

The patients group (Table I) comprised 51 males (67%) and 25 females (33%); ages ranged from 4 to 78 years, with a mean age of 62.6 years.

Hepatocellular carcinoma incidence increased with age, without a significant difference between the two age categories (<65 yrs and ≥ 65 yrs) (p=0.531) (Table I). When the patients were grouped according to age and gender, an inverse distribution was observed. In males, most cases were above the age of 65 (30 patients, 59%), while in females most cases were below the age of 65 (16 patients, 64%). Single tumours were observed in most cases (30 patients, 69.76%). The most affected hepatic segments were 3, 4, 5 and 6 (9 patients for each hepatic segment, each representing 18.3%). With regards to tumour size, 22 patients (29%) were excluded because the tumour size was not available. Thirteen patients presented tumours ranging from 0-3 cm (24%), 7 (13%) had tumours 3-5 cm in size and 34 (63%) had tumours larger than 5 cm. Analysis of resection margins revealed that most presented tumour-free resection margins; 34 patients were biopsied (45% of total) (Table I).

Presence of tumours was associated with HBV or HCV in 30% of cases. Most cases, however, did not have viral infection. HCV infection was more prevalent in females as opposed to males. Regarding tumour differentiation, most cases presented a moderate degree of differentiation (Table I). In most patients with liver cirrhosis, a moderate degree of differentiation was noted (15 patients, 50%) (Table I).

Although we found no statistically significant association between the viral etiology of cirrhosis and the moderate degree of differentiation (p=0.67, OD=1.29, CI95%=0.388-4.343), most HCC cases developed on cirrhotic liver in our patients.

We analyzed the association between the degree of tumour differentiation and the tumour size, after dividing the patients into three categories according to tumour size. Most tumours larger than 5 cm had a moderate degree of differentiation; within the entire group only a few cases presented tumours smaller than 3 cm with a poor degree of differentiation (Table I).

Table I. Patient and tumour characteristics

Variable		No. of patients (%)
Gender	Male	51 (67)
	Female	25 (33)
Age (all)	<65 years	37 (49)
	≥ 65 years	39 (51)
Age (males)	<65 years	21 (41)
	≥ 65 years	30 (59)
Age (females)	<65 years	16 (64)
	≥ 65 years	9 (36)
Number of tumours	unifocal	30 (30)
	multifocal	13 (17)
	unknown	33 (43)
Tumour size	< 3 cm	13 (17)
	3-5 cm	7 (9)
	≥ 5 cm	34 (45)
	N/A	22 (29)
Liver cirrhosis	not associated	48 (63)
	cirrhosis HCV	19 (25)
	cirrhosis HBV	4 (5)
	N/A	5 (7)
Viral liver cirrhosis	not associated	36 (70)
(males)	cirrhosis HCV	10 (20)
	cirrhosis HBV	2 (4)
	N/A	3 (6)
Viral liver cirrhosis	not associated	12 (48)
(females)	cirrhosis HCV	9 (36)
	cirrhosis HBV	2 (8)
	N/A	2 (8)
Resection margins	R0	35 (46)
	R1	7 (9)
	N/A	34 (45)
Grading: total	poor	4 (5)
	moderate	28 (37)
	well	13 (17)
	N/A	31 (41)
Grading: cirrhosis	poor	2 (7)
	moderate	15 (50)
	well	6 (20)
	N/A	7 (23)
Grading: size < 3 cm	poor	1 (8)
	moderate	4 (31)
	well	2 (15)
	N/A	6 (46)
Grading: size 3-5 cm	poor	1 (14)
	moderate	3 (43)
	well	1 (14)
	N/A	2 (29)
Grading: size >5 cm	poor	2 (7)
	moderate	18 (53)
	well	7 (20)
	N/A	7 (20)

N/A: not available; R: resection margin

Analysis of the p53 protein expression

We observed the predominance of a moderate and intense gene expression in our patients (26 cases, 34% and 23 cases, 30%, respectively) (Table II). By analyzing p53 protein expression in accordance with gender, we observed predominance of a moderate protein expression in males (20 cases, 39%), as opposed to females, in whom a low protein expression was more prevalent (11 cases, 44%). We also found that before the age of 65, the majority of cases presented a low protein expression (12 cases, 34% in both categories), but above the age of 65 an intense protein expression was predominant (15 cases, 37%).

Most cases with a moderate degree of differentiation presented a low p53 protein expression (10 cases, 36%). Upon further analysis, most cases in the group presented either low or moderate expression of the p53 protein.

To analyze the correlation between tumour size and enzymatic activity a group of 54 patients was used. Analysis revealed that below a tumour size of 3 cm there were cases with no discernable protein expression (2 cases, 15%); as tumour size increased, so did p53 protein expression. In tumours larger than 5 cm most cases presented an intense p53 protein expression (13 cases, 38%).

The correlation between viral liver cirrhosis and the expression of p53 protein was analyzed. Ten cases (34%) presented low p53 protein expression, 9 cases (30%) moderate expression and 10 cases (34%) intense expression. The results are shown in Table III. None of the risk factors had a statistically significant correlation with the p53 expression.

DISCUSSION

The *p53* gene plays an important role in regulating the cellular cycle, especially in regulating apoptosis. That is why it is considered that tumour behavior can be described using alterations of the *p53* gene, and these are also considered an independent factor in natural disease evolution prognosis [17].

In our study, various levels of the p53 protein expression have been observed in 69 of the 76 patients (91%). The 7 remaining patients (9%) presented no expression of the p53 protein.

Our patient cohort has many similarities to that of Cheng et al. [18], which included 51 males (64%) and 29 females (36 %). Division by age groups was similar, with 33 patients below 60 years (41.25%) and 47 patients over 60 (58.75%). The study analyzed the contribution of certain clinical and paraclinical factors to the occurrence of genetic modifications

Table II. p53 expression evaluated by immunohistochemistry (IHC)

Variable / p53 expression	negative	poor	moderate	intense
Number of patients, n (%)	7 (9)	20 (27)	26 (34)	23 (30)
Gender				
Male, n (%)	4 (8)	9 (18)	20 (39)	18 (35)
Female, n (%)	3 (12)	11 (44)	6 (24)	5 (20)
Age				
< 65 yrs, n (%)	3 (9)	12 (34)	12 (34)	8 (23)
≥ 65 yrs, n (%)	4 (10)	8 (19)	14 (34)	15(37)
Differentiation - grading				
Poor, n (%)	0 (0)	2 (50)	2 (50)	0 (0)
Moderate, n (%)	3 (11)	10 (36)	8 (28)	7 (25)
Well, n (%)	1 (8)	2 (15)	6 (46)	4 (31)
Tumor size				
< 3 cm, n (%)	2 (15)	3 (23)	5 (39)	3 (23)
3-5 cm, n (%)	0 (0)	5 (72)	1 (14)	1 (14)
> 5 cm, n (%)	2 (6)	9 (27)	10 (29)	13 (38)
Presence of cirrhosis, n (%)	1 (2)	10 (34)	9 (30)	10 (34)

in HCC. The most important risk factors, other than the aforementioned gender and age, were HBV infection, cirrhosis, venous invasion, tumour size, degree of tumour differentiation, intrahepatic metastases, lymph node invasion and TNM staging. Considering the risk factors for genetic modification, these results are comparable to our results. Cheng et al. [18] pointed out that the only factors that were associated with significant changes of the p53 gene were the degree of tumour differentiation, lymph node invasion and TNM staging. The other factors were not significantly associated with changes of the *p53* gene.

In our study, with a very similar group, we have reached the conclusion that there was no statistically significant correlation between the analyzed risk factors (viral infection, cirrhosis) and the p53 protein expression.

In the study by Alves et al. [19], 54 Brazilian patients with HCC were included. Of these, 19 (35%) presented immunohistochemical p53 protein expression. From a total of 39 patients with cirrhosis, only 13 (33.3%) presented expression of the p53 protein [19], while in our study, a p53 protein altered expression appeared in 69 patients (91%). The nuclear p53 expression in moderate and high grade of tumour differentiation was detected in 6 out of 27 patients versus 7 out of 12 patients with low tumour differentiation [19]. The difference between the two groups was statistically significant (p=0.03). The authors' conclusion was that p53 expression was intensely seen in very advanced tumours. This correlation suggests that genetic mutations appear only in the advanced phases of the disease. That is why by studying the relation between the presence of viral hepatitis B and C infection and alcohol consumption and

Table III. Analysis of the p53 protein expression by gender, age, viral status, presence of cirrhosis, tumour size

Variable		No. of patients	Odds Ratio	Pearson's chi²/ Kendall's tau-b	p-value	Contingency coefficient
Gender	male	51	0.62	7.17	0.067	0.294
	female	25				
Age	< 65 years	37	0.86	0.067	0.531	-
	≥ 65 years	39				
Age (females)	< 65 years	16	1.14	-0.119	0.486	-
	≥ 65 years	9				
Age (males)	< 65 years	21	4.83	0.108	0.416	-
	≥ 65 years	30				
Tumour size	< 5 cm	18	1.77	-0.136	0.270	-
	≥ 5 cm	36				
HBV or HCV infection	Yes	23	0.95	1.422	0.70	0.241
	No	48				
Hepatic cirrhosis	Yes	30	1.7	2.306	0.511	0.172
	No	46				

the genetic mutations, the researchers noted that there was no significant relation between these risk factors and the genetic mutations. The genetic mutations have been more frequently observed in patients in whom the hepatic tumours did not develop in a cirrhotic liver, as compared to patients who had the tumour developed in a cirrhotic liver [19].

Another study [20] reported an overexpression in 22 cases (31%) detected by immunohistochemistry of protein p53 with monoclonal antigen Pab1801. These mutations were more frequently observed in poor differentiated tumours (p=0.01) and in tumours larger than 5 cm (p=0.05). No statistically significant association was observed with the presence of HBV or HCV infections or with the underlying hepatic conditions. Based on this observation, the authors also reached the conclusion that genetic modifications only appear in the advanced phases of HCC.

Taking into consideration that HCC pathogenesis is a multifactorial process that is not yet completely elucidated, the research continues and besides *p53* gene, other genes that could influence intracellular signaling and finally lead to tumour involution are being presently investigated. The mutations in the *p53* gene are studied not only regarding the intensity of its expression, but also for observing the mutation type in order to gather more information on patient survival. Some studies have shown a significant relation between the intensity of genetic modifications and the patient survival rate.

CONCLUSION

Our study is a promising addition to the research on the p53 protein expression in HCC. As a continuation, the analysis of the relation between the intensity of p53 protein expression and patient survival is proposed, as well as the association between genetic predisposition, lifestyle and intensity of genetic modifications.

Conflicts of interest: Authors declare that they have no conflicts of interest.

Author contributions: F.G., A.R. and L.R. conceived the study. F.G., A.R. and A.B. wrote the manuscript. E.M., A.R. and F.G. made substantial contributions to the acquisition and interpretation of data and drafting of the manuscript. N.H. and V.N. gave the final approval of the version to be published. All authors read and approved the final manuscript

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Analiza expresiei proteinei p53 în carcinomul hepatocelular

ABSTRACT / REZUMAT

Introducere: Carcinomul hepatocelular are o incidență în creștere și studiile cu privire la factorii de risc sau patogeneză pentru acest tip de carcinom beneficiază de un interes deosebit. Acest studiu evaluează corelațiile dintre expresia proteinei p53 si factorii clinici si de laborator la pacienții cu carcinom hepatocelular (HCC).

Metodă: Lotul de studiu include 76 de pacienți diagnosticați cu HCC, prin biopsie sau după rezecția chirurgicală cu scop curativ. Imunohistochimia a fost efectuată la toti pacientii pentru determinarea expresiei proteinei p53. Corelațiile între expresia proteinei p53 și vârsta pacientului, dimensiunile tumorii, prezența infecției virale, și prezența cirozei hepatice au fost efectuate cu ajutorul testului chi-pătrat (Pearson chi-pătrat), împreună cu coeficientul de contingență Kendall (tau-b).

Rezultate: În grupul de studiu, 51 de pacienți au fost de sex masculin (67%) și 25 de sex feminin (33%). Ciroza hepatică virală (cu virus B sau C) (63% din grupul de studiu) nu a fost asociată cu prezența HCC. Expresia proteinei p53 a fost observată la 69 de pacienți (91%). Asocierea dintre expresia proteinei p53 și sexul pacientului ($p = 0.067$), vârsta ($p = 0.531$), mărimea tumorii ($p = 0.270$), prezența hepatitei B și a infecției virale C ($p = 0.7$), prezența cirozei hepatice ($p = 0.511$) nu a fost semnificativă din punct de vedere statistic.

Concluzie: Expresia proteinei p53 nu a fost semnificativ statistic asociată cu caracteristicile demografice ale pacienților, cu mărimea tumorii, cu prezența infectiei virale B și C sau a cirozei hepatice.