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Expression of p53 Protein in Hepatocellular Carcinoma: Relationships to Viral Infection and Prognostic Factors

Thanaa E. Helal[1*] • Monir A. El-Ganzuri[2] • Ahmed M. Aref[3] • Mohamed T. Badr[4]

[1] Department of Pathology, Faculty of Medicine, Ain Shams University, Cairo, Egypt
[2] Department of Zoology, Faculty of Science, Ain Shams University, Cairo, Egypt
[3] Faculty of Dentistry, MSA University, Egypt
[4] Department of Pathology, National Liver Institute (NLI) - Menofiya University, Egypt

Corresponding author: * **thanaahelal@hotmail.com**

ABSTRACT

Hepatocellular carcinoma (HCC) is very common in Egypt due to the high incidence of hepatitis C virus (HCV) infection. The aim of the current study was to assess the relationships between p53 and the viral infection as well as the prognostic factors in patients with HCC. p53 protein was examined by means of immunohistochemistry in tissue sections from 34 HCCs and 16 cirrhotic patients. p53 was expressed in 12 out of 34 HCCs (35%). All the 16 cirrhotic cases were p53-negative. No significant relationship was detected between p53 expression and the prognostic factors ($P > 0.05$). However, the level of p53 expression was significantly higher in patients coinfected with HCV and hepatitis B virus (HBV) than in those having single infection.

Keywords: hepatocellular carcinoma, p53, hepatitis C virus, hepatitis B virus

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm in the world and the third most common cause of cancer-related death; currently, it is the leading cause of death among cirrhotic patients (Llovet *et al.* 2004). It represents more than 5% of all cancers in the world; the estimated number of cancer-related deaths exceeds 500,000 per year (Llovet and Beaugrand 2003).

In Egypt, HCC showed a high relative frequency of 18.1% of digestive system malignancies and 2.6% of total malignancies with a gradual rate of increase (Mokhtar 1991). HCC incidence was estimated to be between 5 and 7/100,000 people/year (Jones 1999). El-Zayadi *et al.* (2005) reported an increase in the proportion of HCC among chronic liver disease (CLD) patients in Egypt with a significant decline of hepatitis B virus (HBV) and a slight increase of hepatitis C virus (HCV) as risk factors. The burden of HCC has been increasing in Egypt with a doubling in the incidence rate in the past 10 years (Anwar *et al.* 2008; Iyer *et al.* 2010).

The two main etiological factors for HCC are cirrhosis and viral hepatitis. HBV and HCV infections as well as aflatoxin B1 ingestion are strongly associated with the occurrence of HCC. Although the precise mechanism underlying the development of HCC is still not clear, it is known that the prevalence of HBV and HCV infections differs relative to the geographical area; HBV is the more predominant agent in liver diseases in South East Asia and Africa, whereas HCV plays a more predominant role in Japan, the United States and Egypt (Michielsen *et al.* 2005; Zekri *et al.* 2009).

During the past decade, p53 has been brought to the forefront of cancer research, and intensive investigation has provided insight into how it mediates its tumour suppressor activities, and how these activities are regulated. Elucidation of the mechanisms that activate and regulate p53, and the identification of upstream and downstream effectors and targets involved in p53 function, should contribute to our understanding of how cancers arise, and to develop more effective new therapeutic tools for their treatment (Ashcroft and Vousden 2001).

p53 is one of the most frequently mutated genes in human cancer, in which loss of function contribute to the development of many major human malignancies. Approximately 50% of all human tumours carry a *p53* mutation, and at least 52 different types of tumour have *p53* mutations. The *p53* gene encodes a 53 kDa protein which plays an essential regulatory role in cell proliferation, it is involved in the control of the cell cycle, DNA replication, DNA repair, differentiation and programmed cell death (apoptosis) (Götz and Montenorh 1995).

In HCC, the prognostic value of p53 is controversial, since several studies show an association with patient survival, while other investigations report no association (Sung *et al.* 2005; Tseng *et al.* 2008).

In the present study liver biopsies from Egyptian patients with HCC were analyzed by immunohistochemistry (IHC) to detect the p53 oncoprotein and to assess the relation of this oncoprotein with other etiologic factors such as cirrhosis, HBV and HCV infections.

MATERIALS AND METHODS

The present study was conducted on 34 tissue specimens of HCC and 16 specimens of liver cirrhosis. These specimens were retrieved from the Pathology Department of the National Liver Institute, Menofia University during the period from 1999 to 2006. The research ethical committee (REC) of Ain Shams University approved the protocol.

The hospital records of these 50 patients were reviewed to record the following data: Patients age and sex, Presence or absence of cirrhosis in the malignant cases as diagnosed radiologically or histologically.

Serologic data

Serum levels of alanine aminotransferase (ALT) (E.C. 2.6.1.2); the cut off value was 40 IU/l.

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1. Serum level of α-fetoprotein (AFP) according to El Zayadi *et al*. (2005); the cut off value was 200 ng/ml.
2. Detection of hepatitis B surface (HBsAg) and hepatitis B core antibody (HBcAb) using the Abbot IMx system (V2) assay, which is based on the microparticle enzyme immunoassay (MEIA) technology according to Shih *et al*. (1980).
3. Detection of HCV-RNA by reverse transcriptase polymerase chain reaction (RT-PCR) as described by Castillo *et al*. (1992).

Hisopathological study

Paraffin blocks from all 50 specimens were retrieved to prepare hematoxylin and eosin stained sections, which were examined histologically for:

A) Confirmation of malignancy in the malignant group and histological grading of the malignant group according to the World Health Organization (WHO) classification (2000) to well differentiated, moderately differentiated and poorly differentiated HCC.
B) Presence or absence of cirrhosis adjacent to non-neoplastic tissue if included in the malignant group.
C) Confirmation of the diagnosis of cirrhosis in the cirrhotic group.

Immunohistochemical study

Sections (5 µm thick) from all 50 paraffin-blocks were cut on positively-charged slides. These slides were examined for p53 oncoprotein expression by means of immunohistochemistry using the primary mouse monoclonal p53 antibody (Biosource, CA, USA) and a detection system (Zymed, San Francisco, USA). The avidin-Biotin-peroxidase technique was preferred according to Hsu and Raine (1981). After deparafinization and rehydration, antigen was retrieved by boiling the tissue sections in 10 mM citrate buffer for 20 min. The tissue sections were incubated with serum blocking solution (0.2% bovine serum albumin) for 10 min, this is important to block non-specific binding. The tissue sections were incubated with the primary antibody in moist chamber for 2 h then incubated with the biotinylated secondary antibody for 15 min then rinsed in phosphate buffered saline (PBS) for 2 min. The tissue sections were incubated with the enzyme conjugate (streptavidin peroxidase) for 15 min and rinsed in PBS for 2 min, then An appropriate amount of the chromogen 3,3' diaminobenzidine tetrachloride (DAB) in a saline solution which contains 0.3% hydrogen peroxide was added to each slide then incubated at room temperature for 30 min and counterstained with haematoxylin, then the sections were examined under a light microscope.

The specificity of the technique was assessed by: a) using a paraffin sections from a case of breast carcinoma known to be positive to p53 as positive control to assess the accuracy of the technique and quality of the reagents. b) For each case studied, serial sections from the specimen were used as a negative control as it followed all the steps of immunostaining except for the step of primary antibody application, which was replaced by application of non-immune serum. The negative control was used to exclude any non-specific staining.

For analyses of the results, positive cases showed brownish nuclear staining. Any case should show nuclear positivity in at least 5% of the tumour cells in order to be considered positive (Paiva *et al.* 2002).

Statistical analysis

The data were coded and processed on an IBM-PC compatible computer using SPSS (v. 11). Chi-square and Fisher's exact test was applied on the malignant group to test the association between the p53 results and the following: patient sex, patient age, serum ALT, α-fetoprotein, HCV-PCR, HBsAg, HBcAb, coinfection with HBV and HCV, grade of HCC, and presence or absence of associated cirrhosis.

RESULTS

The present study dealt with 34 cases of hepatocellular carcinomas. They were 27 males and 7 females (M: F = 3.8: 1). Ages ranged from 30 to 74 years with mean age = 55.4 ± 9.1 years. In addition, 16 cirrhotic cases were included as the control group. They were all males with a mean age of 57.3 ± 8.6 years.

Serologic findings

1. ALT was $\geq$ 40 IU/l in 26 patients, AFP was elevated $\geq$ 200 ng/ml in 14 patients (41.2%).
2. HBV was expressed in 8 patients (23.5%), while HCV infection was confirmed by the presence of HCV-RNA in 31 patients only (91.2%) using RT-PCR technique.
3. Only 6 patients showed coinfection with HBV and HCV (17.6%).

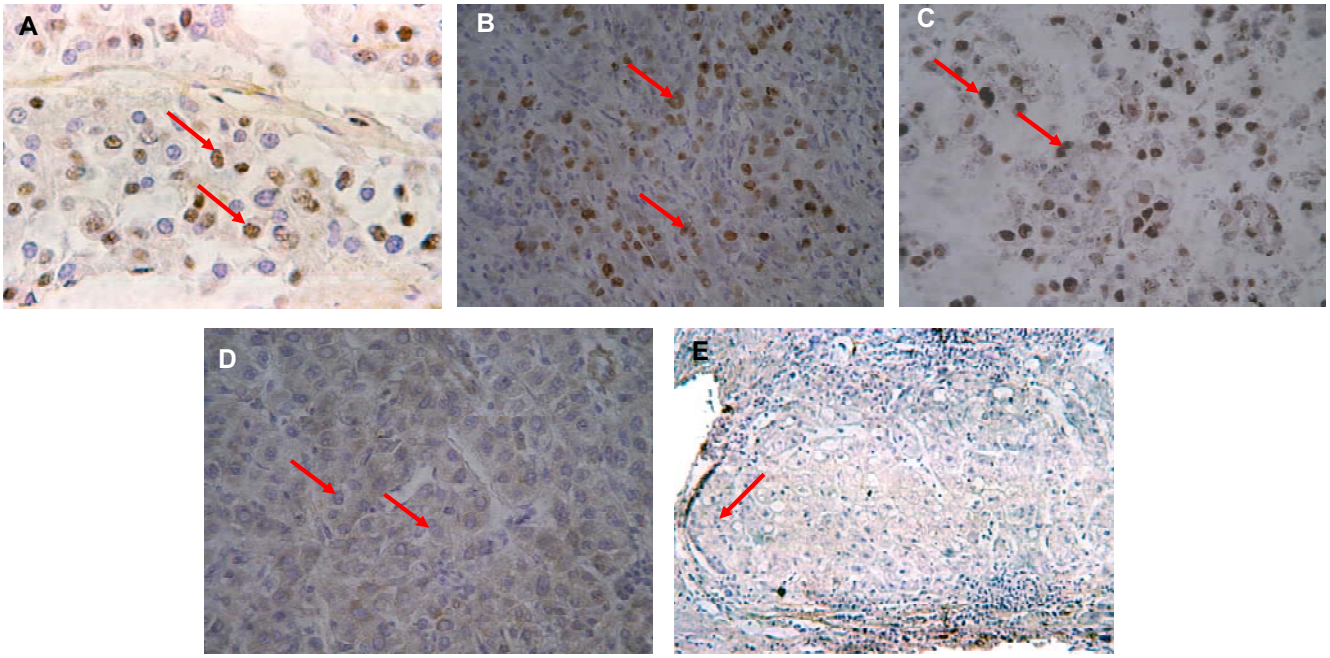

Fig. 1 Nuclear p53 expression in hepatocellular carcinoma. (**A**) Positive expression in grade I (X 400). (**B**) Positive expression in grade II (X 400). (**C**) Positive expression in grade III (X 400). (**D**) Negative expression (X 400). (**E**) Negative expression in cirrhosis (X 250). Arrows point to positive nuclei expressing p53 (brown in colour in **A**, **B**, and **C**) while negative cells are not stained brown (**D**, **E**).

Histopathologic findings

The diagnosed HCC in the 34 malignant cases were graded as follows: Grade I was present in 6 patients (17.6%), grade II was present in 19 patients (55.9%) while grade III was present in 9 patients (26.5%), and 26 out of the 34 cases (76%) were associated with cirrhosis.

Immunohistochemistry results

Twelve out of the 34 malignant cases (35.3%) showed positive p53 immunostaining. They were 1 case grade I (16.7%; **Fig. 1A**), 7 cases grade II (36.8%; **Fig. 1B**) and 4 cases grade III (44.4%; **Fig. 1C**). Ten out of the 12 cases (83%) of HCC with cirrhosis showed p53 expression, while the remaining 22 malignant cases revealed negative p53 expression (64.7%; **Fig. 1D**). All the 16 cases of cirrhosis showed negative p53 immunostaining (**Fig. 1E**).

Relationship between p53 expression and other factors

There was no statistically significant association between p53 results and patients' age, sex, ALT, HCV infection, presence or absence of associated cirrhosis or AFP (**Table 1**). Expression of p53 was significantly higher in HCCs with combined HCV and HBV infection than those with HCV infection only (P = 0.02) (**Table 2**). Although p53 oncoprotein expression was higher in poorly-differentiated than in well-differentiated carcinomas, this difference was not statistically significant.

Table 1 The relationship between p53 expression and other factors.

Factor	p53 +ve	p53 -ve	P
Sex			
Male	10	17	NS
female	2	5	
Age			
≤ 50	6	16	NS
> 50	6	6	
HCV infection			
+ ve	11	20	NS
- ve	1	2	
HCV& HBV			
-ve	7	21	0.02
+ve	5	1	
Tumour grade			
G1	1	5	NS
G2	7	12	
G3	4	5	
AFP			
≤ 200	9	11	NS
>200	3	11	

Table 2 The relationship between p53 expression and HBV/HCV in cases of hepatocellular carcinoma.

p53 expression	HBV/HCV				P
	-ve		+ve		
-ve (22)	21	75%	1	16.7%	0.02 S
+ve (12)	7	25%	5	83.3%	
Total	28	100.0%	6	100.0%	

S: Significant according to Fisher's exact test

DISCUSSION

HCC ranks as the fifth most common cancer in the world; it is also the fifth among men and eighth among women (Parkin 2001; Fabregat 2009; Iyer *et al.* 2010). With an annual incidence of over 660,000 deaths, HCC is the third leading cause of cancer death globally (Huynh 2010). HCC is the second among cancers of the digestive tract after stomach cancer (Bosch *et al.* 1999). HCC develops in patients with CLD, and its etiopathogenesis includes viral infection (hepatitis B and C), alcohol, and aflatoxin B1 consumption. The majority of HCC patients have associated cirrhosis and impaired liver function, making the treatment of HCC more difficult than that of many other cancers (Zhang *et al.* 2009).

There was a remarkable increase in HCC among Egyptian patients with chronic liver disease from 4 to 7.2% over a decade, which reflects the magnitude of the problem of HCC in Egypt (El-Zaydi *et al.* 2005).

The rate of HCC is increasing in Egypt where the major risk factors are chronic infection with hepatitis B and C viruses. Because of the very high prevalence of HCV in the general population, it accounts for the most HCC cases in Egypt (Hassan *et al.* 2002; Ezzat *et al*. 2005). HCC incidence has doubled in Egypt in the past 10 years, which could be attributed to high prevalence of HCV and HBV, although HBV rates have declined after the introduction of the vaccine in 1992 (Iyer *et al*. 2010).

The present study was performed on 34 patients with HCC and 16 cirrhotic liver patients. In this study HCV infection was documented in 93% of patients with HCC. This high rate is consistent with the study of El-Zayadi *et al*. (2005), which was 87.9%, 90% by El-Kafrawy *et al.* (2005), 76.6% by Abdel-Wahab *et al.* (2007), and 77% by Hussien (2004). This data reflects the high incidence of HCV in Egyptian HCC patients.

Despite the significant advances in the knowledge of HCV biology, little is known about the mechanism involved in HCV-associated hepatic carcinogenesis (Rullier *et al.* 2001). Clearly, chronic hepatitis with its association with chronic inflammation, liver cell necrosis, regeneration and extensive fibrosis is a major step in this process (Kew 1989). However, in the present study it was found that 23% of HCC cases were HCV-positive livers without inflammation and cirrhosis and this result supports the hypothesis that HCV can act directly on hepatocytes *via* viral proteins (De Mitri *et al.* 1995).

HBV has also been regarded as a risk factor in the development of chronic hepatitis, liver cirrhosis and primary HCC (Pal *et al.* 2001). However, in the present study it was detected in only 8 cases (23.5%). This result is consistent with the study of El-Zayadi *et al.* (2005), who also only detected HBC in 20.5% of cases, and that of Hussien (2004), in which the incidence was 18.7%. El- Kafrawy *et al*. (2005) detected HBV in only 2.4% of HCC patients.

p53 is an important tumour suppressor gene and its mutation induces cell-dysregulation. HCCs with the p53 mutation had a high malignant potential, and p53 mutation in the primary lesion can be used as an indicator of the biological behaviour of recurrent HCCs, and as an independent prognostic factor affecting survival after recurrence (Nan *et al.* 2005). Several IHC-based studies reported the proportion of p53-positive HCC cases to vary from 22 to 81% (Mann *et al*. 2007). The cause for the variation in p53 expression can be partly attributed to the lack of a consistent cutoff value among different studies for determining positive p53 expression. In some studies, the HCC was regarded as p53-positive when ≥10% of the tumor cells expressed p53, while in others, this cutoff value was defined as ≥5% of the tumor cells being positive for p53; further, the majority of studies have not defined the lower limit for p53-positive tumor cells. Another cause of the discrepancy in the reported percentage of p53-positive tumors is the differences in the p53 expression with the prevalent carcinogenic factors and certain unknown molecular mechanisms (Zhang *et al.* 2009).

Abnormal (the protein has a half-life about 20 min so it is not regularly detected by IHC) immunohistochemical expression of p53 was detected in 35.3% of patients (**Table 3**); this percentage is higher than that obtained by Hussien (2004), who has reported expression in only 25% in Egypt, 15% in Italy (Caruso and Valentini 1999), 19% in Korea (Sung *et al.* 2005), and 29.4% in Japan (Wakasa *et al.* 2007). The higher level of positivity in this study may be due to the use of the anti-p53 clone DO7, which is preferred as it

Table 3 Immunohistochemical expression of p53.

Reference	Country	p53 expression (%)
Skopelitou *et al*. 1996	Greece	47.0
Akyol *et al*. 1999	Turkey	35.0
Altaf 2001	Saudi Arabia	52.0
Paiva *et al*. 2002	Brazil	35.7
Alves *et al*. 2004	Brazil	35.2
Jing *et al*. 2005	China	38.0
Koskinas *et al*. 2005	Greece	53.0
Guo *et al.* 2006	China	33.3
Hu *et al*. 2007	Taiwan	41.9
Tseng *et al*. 2008	Taiwan	37.1
Guo *et al.* 2009	China	48.7
Zhang *et al*. 2009	China	70.7
Current study (2010)	Egypt	35.3

identifies most mutant forms of p53 (Yoshida *et al.* 2004). Our results are in agreement with several international studies (Akyol *et al.* 1999; Paiva *et al.* 2002; Alves *et al.* 2004; Jing *et al.* 2005; Guo *et al.* 2006; Tseng *et al.* 2008) (**Table 3**). However, other studies have reported higher p53 expression (Skopelitou *et al.* 1996; Altaf 2001; Koskinas *et al.* 2005; Hu *et al.* 2007; Guo *et al*. 2009; Zhang *et al.* 2009) (**Table 3**).

The most important objective of our study was to assess the relationship between the p53 expression and the various histopathologic and laboratory data and other possible etiologic factors as cirrhosis, HBV and HCV infections. Similar to many other reports, no significant association could be monitored between p53 expression and each of age, sex, ALT, HCV and the presence of associated cirrhosis (Skopelitou *et al.* 1996; Caruso and Valentini 1999; Alves *et al.* 2004; Sultan *et al.* 2006).

Although our results support the hypothesis that p53 alterations are a late event in hepatocarcinogenesis – as it was detected more frequently in tumours with poor cellular differentiation – it did not reach statistical significance which is similar to other studies (Skopelitou *et al.* 1996; Pannain *et al.* 2004). However, it reached a statistical significance in other reports (Alves *et al.* 2004; Bahnassi *et al.* 2005; Koskinas *et al.* 2005).

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 carcinogenesis (Thomas 1992).

An interesting finding in the current study was the observation that p53 oncoprotein was more expressed in patients coinfected with HBV and HCV than those with HCV infection only. No available studies did such a correlation. It was only reported that p53 revealed comparable expression in HCC for patients with HCV and HBV infections (Koskinas *et al.* 2005).

The significantly higher expression of p53 in HCC patients coinfected with HBV/HCV than those with HCV infection only supports the suggestion that the oncogenic effect of those two viruses may be additive or synergistic (Marrero and Lok 2004). This effect may be exerted through the virally encoded X gene of HBV (HBx). This suggestion is supported by the study of Zhang *et al.* (2006), who speculated that HBx stimulates and influences signal transduction pathway within cells. HBx also binds to such protein targets as p53 to initiate hepatocarcinogenesis. Moreover, Hussain *et al.* (2007) claimed that HBx is frequently mutated. Mutant HBx still retain their ability to bind to p53, and attenuate DNA repair and p53-mediated apoptosis.

In conclusion, the current study emphasizes that HCV and HBV infections are the main risk factors for HCC in Egypt. The significance between p53 protein and coinfection with HBV and HCV suggests that virally-encoded HBx protein has a direct role in hepatocarcinogenesis and this is the main pathway through which it exerts its hepatocarcinogenic effect. HCV exerts its hepatocarcinogenic effect through different pathways including p53 alternations.

The absence of significant relationship between p53 expression and other factors such as histological grade, serum ALT levels, and serum AFP levels suggests that p53 has no prognostic value in patients with HCC. However, further follow-up studies are recommended to confirm this proposal.

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