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Assessment of the Proliferative Marker Ki-67 and p53 Protein Expression in HBV- and HCV-related Hepatocellular Carcinoma Cases in Egypt

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Abstract:

Background: Chronic HBV and HCV infections are the major risk factors for the development of HCC through a multistep pathway that involves viral and non-viral dependent pathophysiological steps. Hepatic expression of the nuclear proliferative marker ki-67 and the p53 oncoprotein were found to be associated with poor outcome. So, the present study was done to evaluate the changes in expression of Ki-67 and p53 oncoprotein, and to determine p53 gene mutation in HBV/HCV-related HCC Egyptian patients.

Methods: Eight HBV-and 22 HCV-positive HCC cases have been examined for the presence of p53 mutation by immunohistochemistry (IHC) and single-strand conformation polymorphism (SSCP), followed by direct DNA sequencing. HCV were genotyped by LiPA-II.

Results: Our results have shown that the proliferative marker ki-67 LI and p53 were highly expressed and significantly related to tumor grade in the Egyptian HCC cases ($p<0.05$). Also, p53 mutation was found in 16 HCC cases by IHC and in 14 HCC cases by SSCP, only 11 patients showed p53 mutation by sequencing. The highest mutation rate was scored for exon 7 (7 mutations) at codon 249; 4 out of 8 (50%) of HBV-related HCC cases and 3 out of 22 (13.6%) of HCV-related HCC cases, followed by exon 5 (3 mutations) at codons 133, 146, 176 in HCV-related HCC cases, then exon 8 at codon 275 in HCV-related HCC cases. The concordance between the IHC and sequencing analysis was 69%.

Conclusion: The present study demonstrates the association between the proliferative marker ki-67 and p53 expression with the tumor grade of Egyptian HBV/HCV-related HCC cases. Our results also support the hypothesis that p53 mutations are rather a late event in the carcinogenesis. Also, they suggest that the final steps of hepatocarcinogenesis are common and independent of the aetiology of the viral infection.

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Introduction

Human liver cancer, primarily hepatocellular carcinoma (HCC), is both common and lethal. It has a poor prognosis worldwide. In Egypt, it is the second most common malignancy in males and the fifth in females. Notable variation in HCC incidence rates worldwide corresponds to the prevalence and pattern of the primary etiological factors. Chronic HBV and HCV infections are the major risk factors for the development of HCC through a multistep pathway that involves viral and non-viral dependent pathophysiological steps [1, 2]. Furthermore, the distribution of the genotype of HCV has also shown geographic characteristics, and an association between genotype-related difference and the severity of liver diseases has been found. In Egypt, there is a high incidence of anti-HCV seropositivity in the population, with an overall age-adjusted prevalence of HCV antibodies of 21.9% [3]. The prevalent genotype in Egypt is type 4, with the presence of other genotypes [4].

Different mechanisms of carcinogenesis are implicated for both HBV and HCV viruses. HBV is a DNA virus that can be integrated in the host genome causing by itself or through proteins chromosomal rearrangements and fixed DNA mutations [5], while HCV, an RNA virus, exerts its oncogenic effects by inducing mutator phenotype the action of its proteins [6]. Chronic hepatitis is characterized by increased regenerative cell proliferation, a process that makes cells more susceptible to gene mutations. Increased DNA synthesis is not sufficient to induce carcinogenesis unless genetic alteration, induced by various factors, appear and gradually accumulate [7]. Recent studies have provided evidence that the p53 tumor suppressor gene plays a major role in hepatocarcinogenesis. The p53 has a critical role for regulation of cell cycle, DNA repair and synthesis as well as in programmed cell death [8]. It is well known that the inactivating mutations of p53 are the most common genetic alterations in human cancers including HCC. The mutational spectrum of p53 has been reported to differ in HCC from different geographical locales such as the G to T transversion at the third position of codon 249 in 30 to 58% of HCC patients in Southern Africa and China, where food is highly contaminated by AFB1 B1 and hepatitis B virus (HBV) infection is endemic [9]. On the other hand, few or none of the mutations occur at codon 249 in low AFB1 B1 exposure areas. The results of several studies regarding the prognostic significance of p53 aberrations in HCC have also varied in different countries. Also, p53 mutation was emphasized in advanced but not in early HCC cases [8]. The detection of p53 mutation has been reported in Egypt on HCC patients infected with HCV virus [10, 11], but there is no report regarding p53 gene mutation by sequencing analysis in HBV infection, in addition to the evaluation of hepatic expression of the nuclear proliferative marker Ki-67 in HCC cases infected with HBV and HCV viruses. So, the aim of this study is: a) To evaluate the changes in expression of Ki-67 and p53 proteins in HCC Egyptian patients infected with HBV and/or HCV viruses, b) to correlate ki-67 and p53 expression with tumor grade, c) to determine the mutational spectrum of the p53 gene in the Egyptian HCC patients infected with HBV and/or HCV viruses, and d) to elucidate its possible role in development of HCC.

Methods

Clinical specimens

This study was performed on fresh tumor specimens and blood samples of 30 Egyptian patients with hepatocellular carcinoma that were obtained at the Surgical Department, National Cancer Institute Hospital, Cairo University, Egypt, during the period from February 1999 to March 2003. The tumor samples were divided into two pieces, one of them was immediately snap-frozen and stored at -800 °C for subsequent DNA and RNA extraction. The other one was fixed in neutral-buffered formalin and processed for routine histological examination and immunohistochemistry. These 30 patients were 18 males and 12 females with the male to female ratio 1.5:1. The age range was 32-77 years with a median age of 46 years. The histological diagnosis of cirrhosis and HCC were based on the internationally based criteria [12]. Twenty-two of the samples included in this study were positive for HCV RNA in serum and tissue by RT-PCR, and 8 cases were positive for HBV by both serological tests using ELISA (Abbott-USA, Chicago, IL, USA) (hepatitis B surface antigen, anti-hepatitis B surface antigen and anti-hepatitis B core) as well as HBV-DNA by polymerase chain reaction (PCR).

Immunohistochemical staining

Monoclonal antibodies to ki-67 (Mab-Mib-1-YLEM, Dako Cytomation, Glostrup,

Denmark) and to p53 protein DO7 monoclonal antibody (Biogenex CA, USA) were used for immunostaining. Five micron-thick sections were cut onto sialinized slides (Positive charge, Optiplus, Biogenex, CA, USA), air-dried overnight at room temperature (RT), incubated at 550 °C for 1 hour, dewaxed in xylene, and rehydrated using graded alcohol concentrations. The antigen retrieval method was done by boiling for 10 minutes in 0.1 M citrate buffer (pH 6) in a microwave oven (600 W). Slides were then incubated for 10 minutes in 0.3% H2O2 to abolish endogenous peroxidase activity and the primary antibodies were added at a 1:50 dilution for 2 hours. The detection was done using the universal labeled streptavidin-biotin method (Vector, Vector Laboratories, UK) according to the manufacturer's instructions. Positive staining was detected with 0.3% 3, 3'-diaminobinzidine tetrahydrochloride in citrate buffer and nuclei were counterstained with Meyer's hematoxylin. Negative controls were obtained by replacing the primary antibody by non-immunized rabbit or mouse serum. The assessment of ki-67 and p53 was based on nuclear staining pattern. At least 10 fields in each tumor section were evaluated at medium power (200X) to determine the proportion of tumor cells and the staining intensity of the entire fields of the sections. The positivity of each staining was also described by means of a positivity index (PI), which indicates the percentage of positive cells among 1000 arbitrarily selected tumor cells in a given tissue section. Ki-67 and p53 were scored positive when 10% of the cells showed nuclear immunostaining. Both p53 and ki-67 were classified as mild (+), 10-25% positive cells; moderate (++), 25-75%; or marked (+++), >75% positive cells [13].

DNA extraction

DNA was extracted from 0.5 to 2.0-g fresh tissue samples according to standard protocols [14].

Detection of hepatitis B virus DNA in the tissues

Polymerase chain reaction amplification of HBV was carried out as described previously by Zekri et al. [15] and 10 µl of each amplicon were analyzed by electrophoresis through an ethidium bromide-stained 1.2% agarose gel.

RNA extraction

RNA was extracted from tumor tissues using SV Total RNA Isolation System (Promega Biotech, Madison, WI, USA).

Detection of hepatitis C virus RNA in the tissues

RT-PCR were performed with a primer pair selected from the highly conserved 5-UTR of HCV genome [16]. All steps were done as previously described by Zekri et al. [17]. The following sequences were used as antisense primers for c-DNA synthesis HCV-6 [5-ACC-TCC nucleotides (NT) 319–324]. The internal primers were RB6A and RB6B for amplification of 265 bp of the 5-UTR, RB6A [5- GTG AGG AAC TAC TGT CTT CAC G-3 (NT 47–68)], and RB6B [5-ACT CGC AAG CAC CCT ATC AGG-3 (NT 292–312)]. All samples were analyzed twice for HCV RNA by the RT-PCR on different days with identical results. Upon the completion of the amplification reaction, 10 µl of each PCR reaction product were analyzed by electrophoresis through a 1.2% agarose gel in Tris-Acetate-EDTA buffer (pH 8.0) and ethidium bromide staining. DNA was transferred from the gel onto nitrocellulose filter using alkaline buffer (4N NaOH). The transferred DNA was cross linked by incubation for 2–3 hr at 80 °C and the blot was then hybridized with an internal probe [17].

Genotyping of HCV

The clinical samples were genotyped with the kit of INNO-LiPA II. The line probe assay was used to assess HCV genotypes using kits provided by INNOGENETICS, N.V. The 5-UTR region was amplified using nested PCR with biotinylated primers. The labeled amplicon was allowed to hybridize and mounted on a strip. After stringent washing, streptavidin labeled with alkaline phosphatase was used to trace the hybridized products, and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate were used as a substrate according to the manufacturer's instructions. The probe reactivity patterns were interpreted using the chart provided by the manufacturer [18].

P53 mutation analysis

The PCR-single strand conformation polymorphism technique (SSCP) was performed

as previously described [19] and used to screen exons 5–8 of the p53 gene. All PCR samples with aberrant conformers on SSCP were then sequenced using the Affymetrix GeneChip technique as previously described [10].

Statistical Analysis

Statistical analysis was done using SPSS program version 11 statistical software package. Chi-square analysis was used for contingency table analysis and Fisher's exact testing proportion independence. Significance levels of ≤0.05 were considered significant.

Results

Genotyping of HCV

Twenty-two HCV-related HCC samples were genotyped with the kit of INNO-LiPA II, and all of these cases were genotype 4.

PCR-SSCP analysis

The PCR-single strand conformation polymorphism technique (SSCP) was used to screen exons 5–8 of the p53 gene in the Egyptian HCC patients infected with HBV and/or HCV viruses. A distinct mobility shift was detected in 14/30 (46.6%) of the cases, of which 11 cases showed p53 mutation sequence analysis. The concordance between the IHC and sequencing analysis was 69%.

P53 mutations in HBV/HCV-related HCC cases

Four out of 8 (50%) of HBV-related HCC cases and 7 out of 22 (32%) of HCV-related HCC cases were found to have p53 mutations. Interestingly, all p53 mutations of HBV-related HCC cases were at exon 7 codon 249.

The highest mutation rate was scored for exon 7 (7 mutations) at codon 249; 4 out of 8 (50%) of HBV-related HCC cases and 3 out of 22 (13.6%) of HCV-related HCC cases, followed by exon 5 (3 mutations) at codons 133, 146, 176 in HCV-related HCC cases, as shown in Fig. 1-A, then exon 8 at codon 275 in HCV-related HCC cases, as shown in Table 2.

Immunostaining results

P53 was highly expressed and significantly related to the tumor grade ($p<0.001$). Analytically, the expression of p53 was detected in 16/30 (53%) of the cases; 0 of 1 (0%) of grade I, 5 of 14 (36%) of grade II, 9 of 13 (69%) of grade III, 2 of 2 (100%) of grade IV, as shown in Table 1 and Fig. 1-B. No differences were seen between HBV- and HCV-related HCC cases regarding p53 expression. Other clinicopathological features of the Egyptian HCC cases in relation to p53 expression were shown in Table 2.

The proliferative marker ki-67 LI was highly expressed and significantly related to tumor grade in the Egyptian HCC cases ($p<0.05$). Analytically, the ki-67 LI was 4.12 ± 4.01 in grade I HCC, 14.62 ± 10.11 in grade II, 23.27 ± 11.03 in grade III, and finally 29.00 ± 15.01 in grade IV ($p<0.001$), as shown in Table 1 and Fig. 2. No differences were seen between HBV- and HCV-related HCC cases regarding ki-67 expression.

Table 1. Expression ki-67 and p53 in relation to tumor grade of HCC cases

Tumor grade	No. (30)	P53 IHC (+) (16/30)	P53 DNA (+) (13/30)	Ki-67 labelling index (mean ±SD of LI)
Differentiation				
I	1	0 (0%)	0	4.12 ± 4.01
II	14	5(36%)	4	14.62 ± 10.11
III	13	9(69%)	7	23.27 ± 11.03
IV	2	2(100%)	2	29.00 ± 15.01
		p< 0.001		p< 0.001

Table 2. Individual immunohistochemical and genetic changes in relation to the clinicopathological features of HBV/ HCV-related 11 HCC patients with p53 mutations

no.	Age	Sex Sex	HBV	HCV	Lymph node	No. of tumor nodule	Micro- satellites	Inflamm. infiltration	tumor grade	SSCP	IHC	Exon	codon
1	44	M	+	-	-	>2	present	Moderate	III	+	+	7	249
2	53	F	-	+	+	>2	present	Moderate	III	+	+	5	176
3	62	M	-	+	-	1	absent	Mild	II	+	-	7	249
4	68	M	+	-	+	>2	present	Severe	III	+	-	7	249
5	49	M	-	+	-	1	present	Mild	II	+	+	8	275
6	31	M	-	+	-	>2	absent	Moderate	III	+	+	5	133
7	48	F	-	+	-	1	present	Mild	III	+	+	5	146
8	66	M	+	-	+	> 2	present	Severe	IV	+	-	7	249
9	51	F	-	+	-	1	absent	Mild	III	+	+	7	249
10	60	M	-	+	+	>2	present	Severe	IV	+	+	7	249
11	67	M	+	-	-	>2	present	Moderate	III	+	-	7	249

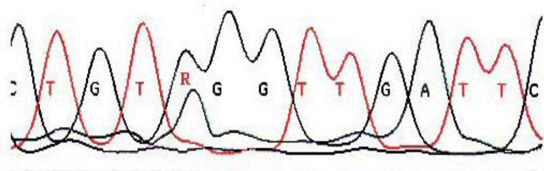

Fig. 1-A. p53 mutation at exon 5 codon 146 TGG- TAG.

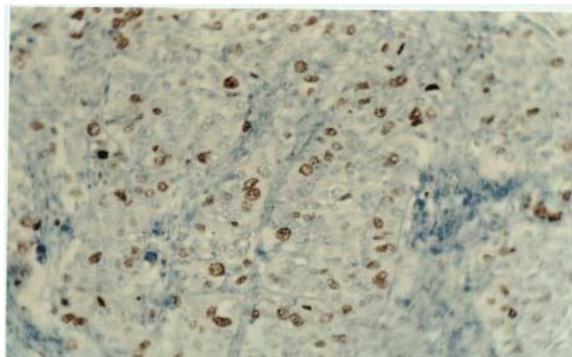

Fig. 1-B. p53 over expression in malignant liver tissue of HCC patients.

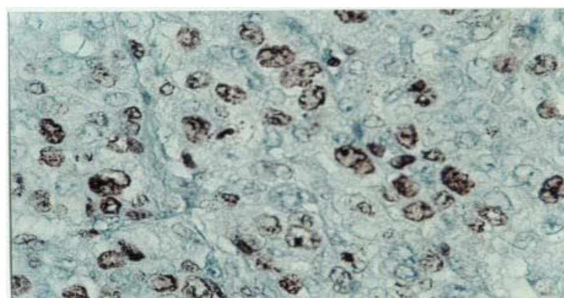

Fig. 2. Expression of the proliferative marker Ki-67 in HCC patients.

Discussion

Hepatocellular carcinoma (HCC) is triggered by many factors including infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV). However, the precise mechanism underlying the development of HCC is still not clear. Recent studies have provided evidence that the p53 tumor suppressor gene plays a major role in hepatocarcinogenesis. The mutations of p53 are common in human HCC, vary considerably in different geographical regions, ranging from 10 to 60% in incidence and have been associated with histological grade, size of tumor and age of the patients [20 ,21].

In the present study, the expressions of the proliferative marker ki-67 and p53 have been evaluated in 8 HBV-related HCC cases and 22 HCV-related HCC cases. Ki-67 and p53 were highly expressed and significantly related to the tumor grade (p<0.001). Both of these ki-67 and p53 markers were found to be significantly higher in advanced stages, portal invasion and intra-hepatic metastasis and were associated with poor outcome [22, 23]. HCC patients that showed P53 over expression had more cirrhosis, larger tumor size, more lymph node involvement, and more microsatellites. No differences were seen between HBV- and HCV-related HCC cases regarding ki-67 and p53 expression. These results are in agreement with koskinas et al. [24] and Ng et al. [25]. The detection of p53 in HCC by immunohistochemistry was found to be related with the presence of mutated inactive p53 protein (26). Our results showed p53 overexpression in 16/30 (53%) of cases. Four out of 8 (50%) of HBV-related HCC cases and 12 out of 22 (54.5%) of HCV-related HCC cases were found to have p53 overexpression. Our findings is close to that reported by Zekri et al. [11] (52%) in Egypt, Koskinas et al. [24] (53%) in Greece, Chen Ban et al. [27] (43%) in China, and Volkmann et al. [28] (45%) in Germany, while this result is different from the other study done by Boix-Ferroro et al. [29] who found p53 overexpression in 20% of HCC Spanish cases. This discrepancy in results could be due to the difference in the sensitivity of the monoclonal antibodies used.

Regarding p53 gene mutation, 11 HCC cases showed p53 mutations. Four out of 8 (50%) of HBV-related HCC cases and 7 out of 22 (32%) of HCV-related HCC cases were reported. The difference between the incidence (16/30 (53%)) of p53 protein overexpression and those that have (11/30 (37%)) p53 genetic alteration could be explained by: i) The presence of other factors that might contribute to the inactivation of the p53 rather than mutations [30], ii) the presence of missence mutation [20], or iii) the threshold values of p53 protein are different [28].

Regarding P53 mutations in HBV/HCV-related HCC cases, all p53 mutations (4/8 (50%)) of HBV-related HCC cases were at exon 7 codon 249. On the other hand, p53 mutations (7/22 (32%)) of HCV-related HCC cases were at exon 7 codon 249 (3 mutations), exon 5 codons 133, 146, 176 (3 mutations), and exon 8 codon 275 (one mutation). Our results support a previous study done by Bressac et al. [31], who stated that a point mutation at codon 249 was common in chronic HBV-related HCC cases. In HCV-related HCC cases, this observation is less common [9], which is in agreement with our results and the previous studies done by Scorsone et al. [32] and Li et al. [33]. However, our results are different from those reported by Oda et al. [34] and Zekri et al. [11]. The discrepancy between the current study and those of the previous studies could be attributed to the different carcinogens that are involved in the cancer etiology and molecular pathogenesis.

In conclusion, the present study demonstrates the association between the proliferative marker ki-67 and p53 expression with the tumor grade of Egyptian HBV/HCV-related HCC cases. Our results also support the hypothesis that p53 mutations are rather a late event in the carcinogenesis. Also, they suggest that the final steps of hepatocarcinogenesis are common and independent of the aetiology of the viral infection.

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