Relationship between codon 249 mutation in exon 7 of *p53* gene and diagnosis of hepatocellular carcinoma

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Abstract

Introduction: Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Multiple genetic and epigenetic changes are involved in the molecular pathogenesis of HCC. Heat shock proteins have essential roles in protecting cells from the potentially lethal effects of stress. Among them, HSP70 are often overexpressed in cells of various cancers and have been suggested to contribute to tumourigenesis. p53 mutations in codon 249 have also been identified in HCC.

Material and methods: Fifty patients with liver disease were enrolled in this study compared to 10 healthy volunteers. The studied patients were divided into 2 groups: group I includes those suffering from HCC, group II includes those suffering from post-hepatitis B and C liver cirrhosis. The presence of *p53* gene mutation was detected by DNA extraction from whole blood of patients and controls followed by polymerase chain reaction then restriction fragment length polymorphism (RFLP) analysis of codon 249 of exon 7. We also studied the genotypes of the *HSP70* gene by PCR followed by RFLP analysis.

Results: Our results revealed no statistical difference between group I, group II, and the control group as regards exon 7 mutation of the *p53* gene. Also the frequency of polymorphic genotypes of HSP70 showed no significant difference between the 3 studied groups.

Conclusions: The present study supports the view that the incidence of point mutation of p53 codon 249 mutations in exon 7 of the *p53* gene may not play a role in carcinogenesis of HCC in Egyptian patients. Also, genetic polymorphism in HSP70 was not associated with high risk of future development of HCC.

Key words: hepatocellular carcinoma, p53, HSP70, hepatitis C virus, hepatitis B virus.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and is estimated to cause over one million deaths every year. Risk factors include chronic hepatitis B and C virus infections and exposure to aflatoxin B1. Chronic hepatitis B virus (HBV) and hepatitis C virus HCV infections are the major risk factors for the development of hepatocellular carcinoma (HCC) through a multistep pathway that involves viral and non-viral-dependent pathophysiological steps [1].

Besides being useful in identifying people at risk for a genetically based disease, knowledge of the polymorphisms that cause disease can provide valuable insight into how the disease develops [2].

Mutation or deletion of the *p53* gene, which plays an important role in cell growth, division and apoptosis by acting as a transcription factor or by forming complexes with other proteins, is frequently detected in HCC. In addition, loss of the functional *p53* gene is associated with lower cellular differentiation and poor prognosis [3].

Abnormalities of p53 are also considered a predisposing factor for hepatocarcinogenesis. Hence, restoration of a wild-type *p53* gene is an attractive approach to the treatment of HCC [4].

p53 mutations have been identified in several human cancers. A selective G to T transversion mutation at codon 249 (AGG → AGT, arginine to serine) of the *p53* gene has been identified as a "hotspot" mutation for hepatocellular carcinoma (HCC) in the Qidong area [5].

Heat shock proteins (HSPs) are overexpressed in a wide range of human cancers, and are implicated in tumour cell proliferation, differentiation, invasion, metastasis, death, and recognition by the immune system [6].

The incidence of hepatocellular carcinoma (HCC) is increasing worldwide and accounts for as many as 1.2 million deaths annually, especially in Asia and Southern Africa. Most HCCs are associated with chronic liver diseases resulting from hepatitis B or C viral infection, and the processes of chronic inflammation and fibrosis act as a stressful condition. Heat shock proteins induced in response to this stressful condition may contribute to hepatocarcinogenesis. Until now there have been a few comprehensive studies of the expression of HSP70 in HCC; however, its prognostic relevance remains controversial. In addition, there have been few studies on HSP70 expression in association with tumour cell proliferation or apoptosis in HCCs [7].

The objective of this work is: to clarify the relation between the selective mutation resulting in a serine substitution at codon 249 in exon 7 of the *p53* gene among patients with hepatocellular carcinomas and patients with cirrhosis, occurring in populations with viral hepatitis B or C infections in Egypt; and also to investigate whether polymorphism in the heat shock protein 70 (HSP70) gene has any bearing on individual susceptibility to the development of HCC.

Material and methods

The current study was conducted on 50 patients with liver disease. They were admitted to the Tropical Department of Kasr El Aini Hospital during the year 2007. The study was approved by the ethical committee. The studied patients were divided into 2 groups: group I (HCC) – included 20 subjects suffering from HCC, 17 males (85%) and 3 females (15%), aged between 42 years and 70 years with a mean of 61.9 ±7.07; group II (LC) – included 30 subjects suffering from post-hepatitis B and/or C liver cirrhosis, 14 males (46.7%) and 16 females (53.3%), aged between 36 years and 77 years with a mean of 52.2 ±9.4. Control group: comprised 10 healthy volunteers not suffering from any liver disease. They were 6 males (60%) and 4 females (40%) aged from 50 years to 65 years (mean of 59 ±4.76 years).

Diagnosis included clinical, laboratory and radiological findings. The controls were recruited from outpatient clinics among individuals with no history or clinical findings suggestive of liver disease, and having the same distributions of age, gender and recruitment site as the HCC and LC (liver cirrhosis) patients.

Determination of p53 mutation

DNA extraction: 200 µl of whole EDTA blood was used for genomic DNA extraction using QIAamp DNA Mini Kit (Catalogue No. 51104), QIAGEN.

DNA amplification and PCR analysis of exon 7

The PCR primers used were as follows:
Primer 1 (up) (5'-CTT GCC ACA GGT CTC CCC AA-3'), Primer 2 (down) (5'-AGG GGT CAG CGG CAA GCA GA-3').

All reactions were performed in a total volume of 50 µl containing: 25 µl Taq Master Mix (QIAGEN).

The thermocycling procedure was done in a PE 9700 (Perkin-Elmer, Foster City, CA) with an initial denaturation step of 5 min at 94°C followed by 40 cycles of 94°C for 30 s, annealing at 60°C for 30 s and 72°C for 30 s, and finally extension at 72°C for 10 min. The 254-bp long amplified PCR products were electrophoresed on 4% agarose gel, stained with ethidium bromide, and visualized by a UV transilluminator.

Mutation detection by restriction analysis

The 254 bp extracted DNA fragment, derived from exon 7 of the *p53* gene, was submitted to restriction enzyme Hae III (BsuRI) from Fermentas Life Science.

A mutation at codon 249-250 will result in an uncleaved 158-bp fragment, and this feature will be distinguished from that of normal samples generating 92 bp, 66 bp and several small fragments from the 254 bp amplified DNA PCR product. Electrophoresis on 4% agarose gel stained with ethidium bromide will distinguish positive cases (with 249 mutation) from negative ones and controls (wild-type). The samples were then visualized on a UV transilluminator (Figures 1, 2).

Determination of *HSP70* gene polymorphism

The same steps as previously done for p53, but using the following different primers for HSP70-1 and HSP70-2:

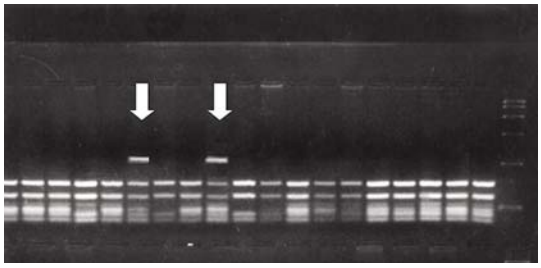

Figure 1. Gel electrophoresis (4%) showing exon 7 mutation of *p53* gene by PCR-RFLP technique using restriction enzyme HaeIII revealed the following: lanes 1, 2, 3, 4 and 5 – negative controls; lanes 6 and 9 – positive cases of HCC samples revealing uncleaved 158 bp, 92 bp, and 66 bp fragments denote heterozygous mutation; lanes 7, 8, 10 → 19 are negative cases of group I and II revealing 92 bp and 66 bp fragments denoting wild type p53

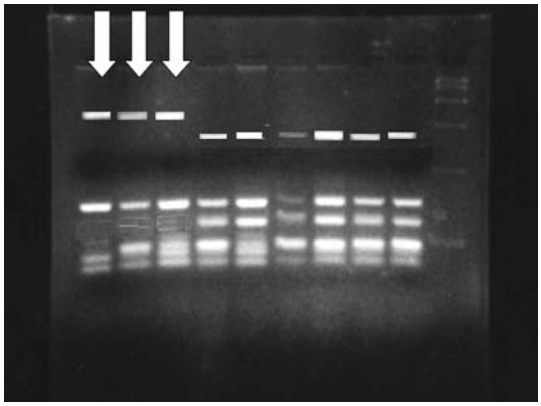

Figure 3. Characterization of BseNI polymorphism of the HSP70-1 gene. Ethidium bromide stained gel of the PCR products digested with BseNI. Amplification of genomic DNA from different individuals containing the 2 possible genotypes is shown. Lane 10 is the DNA marker (50 bp), lanes 1, 2, 3 represent b2/b2 genotype, 2 bands of 84 and 241 bp, lanes 4, 5, 6, 7, 8 and 9 represent b1/b1 genotype, 3 bands of 70, 84, 171 bp

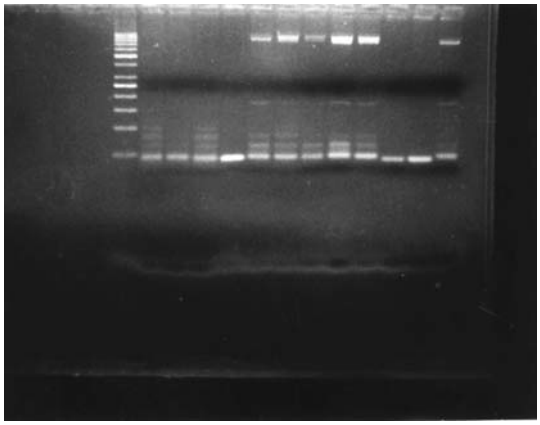

Figure 4. Ethidium bromide stained gel of the PCR products digested with PstI of the HSP70-2. Individuals containing 3 different possible genotypes are shown: lane 1 is the DNA marker (50 bp), lanes 6, 7, 8, 9 and 10 represent AB genotype, 3 bands of 1117, 936 and 181 bp, lanes 5, 11 and 12 represent AA genotype, one band of 1117 bp, lane 13 represents BB genotype, 2 bands of 936 and 181 bp

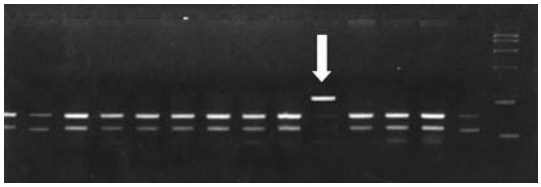

Figure 2. Homozygous mutation of exon 7 of *p53* gene stained with ethidium bromide on 4% gel. Mutation at codon 249 was identified by restriction enzyme HaeIII. Lane 10 is the only HCC case revealing an uncleaved, 158 bp fragment

HSP70-1

The PCR primers used were as follows: (Forward) 5'-TCCGGCGTCCGGAAGGACC-3', (Reverse) 5' -TGCGGCCAATCAGGCGCTT-3 '.

Mutation detection by restriction analysis using: the biallelic polymorphism at position 190 in the *HSP70-1* gene was detected by BsrBI restriction enzyme digestion of the fragment of 325 bp DNA which was previously amplified by the above-mentioned primers. The restriction enzyme BseNI (BsrBI) used was from Fermentas Life Science.

The presence of the 171 bp, 84 bp and 70 bp fragments represents the HSP70-1*b1 allele, whereas the b2 allele showed only 2 fragments of 241 bp and 84 bp. Electrophoresis on 4% gel and staining with ethidium bromide distinguishes the 2 genotypes from each other (Figure 3).

HSP70-2

The PCR primers used were as follows: an HSP70-2 restriction fragment length polymorphism at position 1267 was characterized by a PCR procedure. This analysis was performed considering the polymorphic PstI site at position 1267 of these alleles. We used the following primers spanning the polymorphic PstI site (nucleotide 1267) according to the following described sequence, sense (nucleotide 1083-1102 within the coding region) 5'-CATCGACTTCTACACGTCCA-3' and antisense (nucleotide 2180-2199 within the 3' untranslated end to avoid HSP70-1 homology) 5'-CAAAGTCCTTGAGTCCCAAC-3'.

Mutation detection by restriction analysis

The same steps as previously described but using the restriction enzyme PstI from Fermentas Life Science.

Detection of the PCR RFLP products using 4% gel electrophoresis stained with ethidium bromide and ultraviolet light transillumination. The DNA-lacking polymorphic PstI site within the *HSP70-2* gene generated a product of 1117 bp after restriction (A allele), whereas the PstI site produced 2 fragments of 936 bp and 181 bp (B allele) (Figure 4).

Statistical analysis

Data were collected, revised, verified then edited on a PC. Data were then analysed statistically using SPSS statistical package version 13. Comparisons between groups were made using *t*-test and χ^2 test. A value of $p < 0.05$ was considered statistically significant.

Results

Mean and SD of liver function tests in groups I (HCC) and II (liver cirrhosis) is showed in Table I, comparison between groups I (HCC) and II (liver cirrhosis) as regards positive hepatitis markers in Figure 5, and α-fetoprotein (AFP) (ng/dl) in the 3 studied groups in Table II.

Codon 249 mutation in exon 7 of *p53* gene

Regarding mutation of codon 249 in exon 7 of the *p53* gene using PCR-RFLP technique, our work gave the following results: among the controls, no point mutation at codon 249 of exon 7 was detected. Group II (liver cirrhosis) also revealed no mutation at that point. In group I (HCC), 3 out of 20 patients (15%) had this G to T transversion at the third base of codon 249 of the *p53* gene, whereas 17 patients (85%) were wild type (Figure 1).

One of the 3 mutated patients was homozygous for the mutation (both alleles of the *p53* gene were mutated) while the other 2 patients were heterozygous for the same mutation (Figure 1 – lanes 6 and 9 positive cases; heterozygous mutation; Figure 2 – lane 10 positive case; homozygous mutation).

Patients showing the mutations were males only, but we cannot consider it as a difference, because of the small number of females included in the study group (3 out of 20).

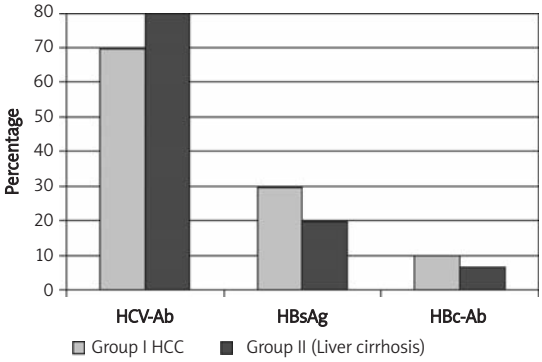

Figure 5. Comparison between groups I (HCC) and II (liver cirrhosis) as regards positive hepatitis markers

Table I. Mean and SD of liver function tests in groups I (HCC) and II (liver cirrhosis)

	HCC (n = 20)	Cirrhosis (n = 30)	Value of p
ALT [U/l]	82.4 ±46.37	34.73 ±22.56	< 0.001**
AST [U/l]	113.15 ±77.12	51.9 ±27.6	< 0.001**
T-Bili [mg/dl]	1.73 ±1.96	2.45 ±3.44	> 0.05
D-Bili [mg/dl]	0.97 ±1.71	1.25 ±2.33	> 0.05
T-protein [g/dl]	7.03 ±0.83	7.17 ±0.99	> 0.05
Albumin [g/dl]	3.09 ±0.59	3.03 ±0.70	> 0.05
GGT [U/l]	139.8 ±123	44.96 ±31.5	< 0.001**
AFP [ng/dl]	387.98 ±539.45	6.32 ±3.81	< 0.001**
PT [%]	80.25 ±9.4	76.89 ±13.2	> 0.05

*p > 0.05 – non-significant, *p < 0.05 – significant, **p < 0.001 – highly significant*

Table II. α-Fetoprotein (AFP) in the 3 studied groups

	AFP [ng/dl]		Value of p
	Range	Mean ± SD	
HCC group (n = 20)	15.5-2477	378.9 ±539.4	< 0.001**
Cirrhosis group (n = 30)	0.8-15.3	6.32 ±3.81	
HCC group (n = 20)	15.5-2477	378.9 ±539.4	< 0.05*
Controls (n = 10)	1.3-5.1	1.3-5.1	
Cirrhosis group (n = 30)	0.8-15.3	6.32 ±3.81	> 0.05
Controls (n = 10)	1.3-5.1	1.3-5.1	

*p > 0.05 – non-significant, *p < 0.05 – significant, **p < 0.001 – highly significant*

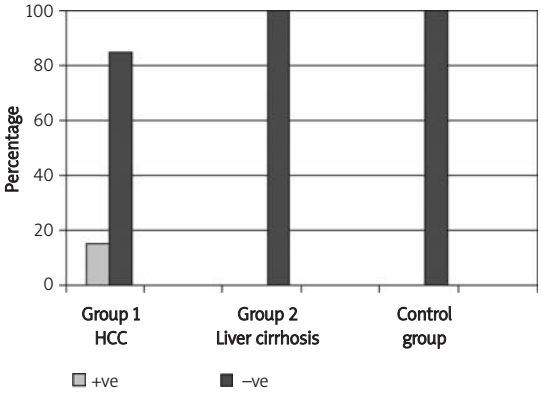

Figure 6. Comparison between the 3 groups as regards exon 7 mutation

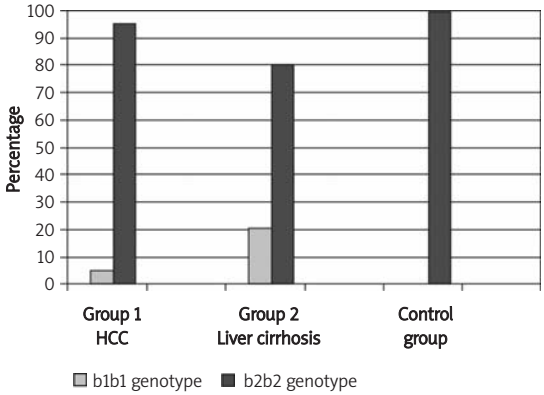

Figure 7. Comparison between 3 groups as regards HSP70-1 genotypes

Patients with 249^{ser} mutation showed no statistically significant difference in mean age from those without the mutation (p = 0.22) (Figure 6).

Polymorphism of HSP70-1

The polymorphism of HSP70-1 was examined and revealed the following genotype frequency in Egyptian patients in group I (HCC) and group II (liver cirrhosis) and in healthy controls (Figure 7).

The frequency of polymorphic genotypes showed no significant difference between the 3 studied groups (p > 0.05), although the HSP70-1 b2b2 genotype is dominant in the 3 groups.

Polymorphism of HSP70-2

The genotype frequencies of individuals homozygous for allele A (AA), heterozygous for alleles A and B (AB) and homozygous for allele B (BB) among the 3 studied groups are shown in Table III.

The frequency of polymorphic genotypes showed no significant difference between the 3 studied groups (p > 0.05):
HSP70-2 A/B and B/B genotypes between group I and control OR 3 (95% CI = 0.606-14.86),
HSP70-2 A/B and B/B genotypes between group II and control OR 3.5 (95% CI = 0.753-16.263).

Discussion

The current study was conducted on 50 subjects with liver disease compared to 10 age- and sex-matched healthy volunteers representing the control group. The study group was subdivided into group I that included 20 subjects suffering from HCC and group II that included 30 subjects suffering from post-hepatitis B and/or C liver cirrhosis.

Our study revealed that in group I (HCC), 14/20 patients (70%) were seropositive for HCV-Ab, and in group II (liver cirrhosis), 24/30 (80%) were seropositive for HCV-Ab.

Hassan *et al.* [9] estimated the risk of HCC in relation to HCV and HBV in Egypt. Thirty-three patients with HCC and 35 healthy controls who had a similar socioeconomic status were prospectively enrolled. They estimated the attributable fraction of HCC to HCV to be 64% in the studied population and 48% in the general Egyptian population. They concluded that both HCV and hepatitis B virus infection increase the risk of HCC in Egyptian patients. However, because of the very high prevalence of HCV in the general Egyptian population, it accounts for most HCC cases in Egypt.

Chronic HBV infection is one of the most important risk factors for HCC in humans. It is estimated that 80% of HCC worldwide is aetiologically associated with HBV. In the United

Table III. Comparison between 3 groups as regards HSP70-2 genotypes

HSP70-2	HCC (n = 20) No. (%)	Liver cirrhosis (n =30) No. (%)	Control (n = 10) No. (%)
Genotype			
A/A	0	3 (10%)	0
A/B	5 (25%)	6 (20%)	5 (50%)
B/B	15 (75%)	21 (70%)	5 (50%)
Alleles			
A	5 (12.5%)	12 (20%)	5 (25%)
B	35 (87.5%)	48 (80%)	15 (75%)

States, although the infection rate is low, HBV is estimated to account for one fourth of HCC cases among non-Asians [10]. In our current study HBsAg was present in 30% of the patients in group I (HCC) and in 20% of group II with liver cell cirrhosis ($p > 0.05$).

Mutation or deletion of the *p53* gene, which plays an important role in cell growth, division and apoptosis by acting as a transcription factor or by forming complexes with other proteins, is frequently detected in HCC. Codon 249 of p53 is a hotspot for AFB1 modification and AFB1-induced mutation, specifically AGGC → AGTC. This mutation has been found in up to 50% of HCC samples in areas of the world where aflatoxin exposure is high. To date, over 2000 HCC samples from all over the world have been examined for this mutation [3]. Kirk *et al*. [11] reported that, in regions where exposure to aflatoxin is high, namely in Qidong and Tongan (China), India, Southern Africa, Gambia, Mozambique and Senegal, 116 out of 262 (44%) of the total HCC cases examined showed a predominance of GC TA mutations at the third position of codon 249 of the *p53* gene. In contrast, in regions of low exposure to aflatoxin, namely Australia, Europe, Japan and the USA, only 17 out of 1273 (1%) of the HCC samples examined had mutations at this site [12].

Sohn *et al*. [13] demonstrated that in regions where exposure to aflatoxin is moderate – namely Beijing, Shanghai, Hong Kong, Singapore, South Korea, Taiwan, Southern Asia, South Africa and Egypt – 40 out of 568 (7%) HCC cases examined had mutations at the third position of codon 249 of the *p53* gene. This p53 mutation appears to be unique to AFB1-induced liver tumours, as tumours presumably induced by other factors fail to consistently show this genetic change.

In our study, examination of the DNA extracted from whole blood of the 50 studied Egyptian patients, as regards mutation of exon 7 of the *p53* gene using the PCR-RFLP technique, revealed that in group II (liver cirrhosis) and the control group no point mutation at codon 249 of exon 7 could be detected, while in the HCC group 3/20 patients (15%) showed the mutation at codon 249 of exon 7. These results were in agreement with another study done in Egypt by Mona *et al*. [14], who reported that in the control group, 20 subjects had no point mutation at codon 249 of exon 7 while in the HCC group out of 30 patients 4 patients (13.3%) showed a point mutation at codon 249 of exon 7 (in both tissue and blood samples). They also reported that out of 30 HCC patients 13 patients (43.3%) showed loss of heterozygosity at codon 72 of exon 4 of the *p53* gene. This finding was in both tumourous tissue samples and the whole blood samples of the same HCC patients.

Tumour-specific mutations of the *p53* gene often lead to synthesis of a faulty protein that has lost its normal growth regulatory functions. The mutation of the *p53* gene in HCC suggests that loss of normal gene function may be a key step during malignant transformation of hepatocytes. In our study, most patients with the 249^{ser} mutation have a corresponding wild-type allele. One of 3 patients was homozygous for the mutation (both alleles of the *p53* gene were mutated), while the other 2 patients were heterozygous for the same mutation. This was in agreement with Mariana *et al*. [15], who suggested that loss of the wild-type allele may be required for the mutant to exert its effect. This observation may support the hypothesis that 249^{ser} mutations are recessive and do not have the dominant negative function reported for some other p53 mutations.

As regards the relation between exon 7 mutation and viral markers, our study showed that among the 14 HCV-Ab seropositive HCC patients there were 3 (21.4%) showing mutation at codon 249 of exon 7 of the *p53* gene in their blood sample. Our results also demonstrated 2 patients with combined HCV and HBV in group I HCC patients, 1 of whom showed exon 7 mutation (50%).

Several reports have documented that prior HBV infection, characterized by presence of anti-HBc, affects the development of HCC in patients infected with HCV. Other authors, however, were not able to document any adverse event of occult HBV infection on the clinicopathological course of chronic HCV infection [16]. On the other hand, our study cannot clarify the fact, as it only included 2 patients out of 30 who suffer from HCC and showed HCV-Ab and HBcAb in their sera. Their clinical data showed no difference compared to other cases with HCC and lacking this coinfection.

Most HCCs are associated with chronic liver diseases resulting from hepatitis B or C viral infection, and the processes of chronic inflammation and fibrosis act as a stressful condition. Heat shock proteins induced in response to this stress condition may contribute to hepatocarcinogenesis. Until now there have been a few comprehensive studies of the expression of HSP70; however, its prognostic relevance remains controversial. In addition, there have been few studies on HSP expression in association with tumour cell proliferation or apoptosis in HCCs. However, no prospective epidemiological data evaluating this gene family are available.

Three of the HSP70 are mapped within the major histocompatibility complex (MHC) class III region on the short arm of chromosome 6. These are intron-less HSP70-1, HSP70-2 and HSP70-Hom, which have a homologous gene sequence but differ in their regulation. There are 14 polymorphic sites

within these 3 MHC-linked HSP70: 6 in HSP-1 and 4 each in HSP-2 and HSP-Hom.

Our aim was to investigate whether polymorphism in the gene for heat shock protein 70 (HSP70) has any bearing on individual susceptibility to the development of hepatocellular carcinoma (HCC). The genotypes of HSP70 genes 1 and 2 of our patients were analysed using polymerase chain reaction followed by restriction fragment length polymorphism.

HSP70-1 b2-b2 genotype frequency was dominant in the control group (100% b2-b2), as well as in the HCC group (95% b2-b2; 20% b1-b1) and in cirrhotics (80% b2-b2; 20% b1-b1). There was no statistically significant difference between the 3 groups regarding the frequency of the polymorphic genotypes ($p > 0.05$). Our results suggested that genetic polymorphism in HSP70-1 is not associated with the risk of future development of HCC in Egyptian patients.

HSP70-2 B/B genotype frequency was increased in the whole group of patients. The A/A genotype was the least frequent among the 3 groups. The B allele was increased among all the groups. In the HCC group the genotype frequency was A/A 0%, A/B 25% and B/B 75%. Among cirrhotics, it was 10%, 20% and 70%, respectively. In the control group the genotype frequency was 0%, 50% and 50%, respectively. The frequency of polymorphic genotypes showed no significant difference between the 3 studied groups as regards HSP70-2 ($p > 0.05$).

Chuma *et al.* [17] compared expression profiles among early components and progressed components of nodule-in-nodule-type HCCs and their corresponding non-cancerous liver tissues with oligonucleotide array. Of these genes, the most abundantly upregulated gene in early HCC components was heat-shock protein 70 (HSP70). Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) confirmed this finding. Further immunohistochemical examination of HSP70 revealed its significant overexpression in early HCC compared with precancerous lesions and in progressed HCC compared with early HCC. Thus, molecular signatures were clearly different in non-cancerous liver tissue as compared with the early and progressed components of nodule-in-nodule-type HCC. Moreover, HSP70 could be a sensitive marker for the differential diagnosis of early HCC from precancerous lesion or non-cancerous liver, a difficult distinction for pathologists due to very well differentiated histology with little atypia in early HCC. Expression of HSP70 may play an important role in hepatocarcinogenesis, and in particular HSP70 can contribute to tumour progression by promoting tumour cell proliferation in HCC [7].

In conclusion, the present study supports the view that the incidence of point mutation of p53 codon 249 is low among Egyptians. The heterozygous mutation of p53 exon 7 found in these patients only indicates that they have genetic susceptibility to HCC. Our results also suggested that genetic polymorphism in HSP70-1 and HSP70-2 was not associated with the risk of future development of HCC in Egyptian patients.

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