Heat Shock Protein-70 and -27 Expressions as Parameters of Early Diagnosis and Disease Progression in Hepatocellular Carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Despite remarkable advances in diagnostic and therapeutic techniques, the incidence of HCC is still on the increase. The role of liver biopsy in diagnosis of HCC has declined. However, with recent advances in genomics and proteomics a great number of potential serum and tissue markers have been identified and are being developed as new candidate markers for both diagnosis and prognosis of HCC, and may increase the need for liver biopsy. The aim of this study is to investigate the role of HSP70 and HSP27 expressions in early detection of HCC and to find their relation to parameters of disease progression. The study was conducted on 76 patients, 42 have proved HCC and 34 patients with liver cirrhosis (LC) without HCC. Routine laboratory investigations were done including: liver function tests, complete blood counts, serum alpha fetoprotein (AFP), hepatitis B and HCV hepatitis markers, and HCV-RNA levels. HSP70 and 27 expressions were determined in liver sampling by Real-Time PCR. Overexpression of HSP70 was detected in 92.86% of HCC which is statistically significantly higher compared to LC (2.94%) cases, and overexpression of HSP27 was significantly increased in HCC cases (57.14%) as compared to LC patients (8.82%). The overexpression of HSP70 was associated with early HCC diagnostic parameters (tumour size) and prognostic criteria (vascular invasion and tumour grade, Overexpression of HSP27 was associated with tumor size and tumor number, but not associated with each of AFP, vascular invasion and tumor grade. Conclusion: From the above results, we conclude that, found expressions of HSP70 and HSP27 may play an important role in hepatocarcinogenesis, and especially HSP70 can contribute tumor progression. We thus suggest that HSP70 may represent a good molecular target for treatment of HCV-related HCC.

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1. Introduction

Stress or heat shock proteins (HSPs) are a set of highly conserved proteins with an essential defense mechanism for protecting cells from various environmental damages **[1].** HSPs have been classified into six major families and designated nomenclature according to their approximate molecular weight: HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs including HSP27 **[2]**.

The HSPs are inducible in response to various physiologically or pathologically stressful conditions, including carcinogenesis **[3]**. The functions of the HSPs in the tumorigenesis have been implicated in the regulation of cell cycle progression and apoptosis **[4]**, in multidrug resistance **[5]**, and as a modulator of p53 function **[6]**.

HSP70 is a housekeeping gene that assists with a variety of vital intracellular Chaperoning functions. Expression of HSP70 increases under conditions of environmental cellular stress **[7]** and overexpression of HSP70 also leads to significant protection against cell apoptosis **[8]**. HSP70 levels block the apoptotic pathway at different levels. HSP70 reduces or blocks caspase activation and suppresses mitochondrial damage and nuclear fragmentation **[9].** HSP70 can also prevent caspase-independent apoptosis pathways. HSP70 prevents cell death under conditions in which caspase activation does not occur, because of the addition of exogenous caspase inhibitors **[10]**.

There were several reports about HSP70 expression in malignant tumors, such as breast cancer **[11]**, lung cancer **[12]**, prostate cancer **[13]**, and carcinoma of the uterine cervix**[14]**. The majority of the published results demonstrated HSP70 overexpression correlated with poor prognosis and resistance to therapy **[15-18]**. HSP27 belongs to the small heat shock protein family. Its structure and function are thought to be modulated by phosphorylation mediated by MAPK2. Intracellular HSP27 plays an anti-apoptotic role through

interaction with Bid or cytochrome c **[19]** and also has a main role as a chaperone, preventing the aggregation of misfolded proteins. As such, HSP27 may contribute to the pathogenesis of human diseases such as cancer, autoimmune diseases, neurological disorders (e.g., Alzheimer's disease), and cardiovascular diseases, where HSP27 has been investigated as a biomarker for myocardial ischemia **[20]**. Increased levels of HSP27 were detected in a number of cancers, especially hormone-sensitive neoplasm **[21]**. Previous studies about the clinical implications of HSP27 showed different results according to tumor types **[21, 22]**.

Hepatocellular carcinoma (HCC) is one of the world's most common malignancies, especially in Asia and southern Africa. Most HCCs are associated with chronic liver diseases resulted from hepatitis B or C viral infection, and the processes of chronic inflammation and fibrosis act as a stressful condition. HSPs induced in response to this stress condition may contribute to hepatocarcinogenesis **[22, 23]**.

Over- expression of 4 members of the HSP family in hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC) samples by proteomic analysis was reported **[24]**. HSP70 is one of these 4 members and displayed the most increased levels in cancerous tissues compared to levels in corresponding noncancerous liver tissues by proteomic analysis. There have been a few comprehensive studies of the expression of HSP70 or HSP27 in HCC **[22, 25]**; however its prognostic relevance remains controversial. In addition, there have been few studies on HSP expressions in association with tumor cell proliferation or apoptosis in HCCs.

Aim of the study:

This aimed to investigate the expressions of HSP70 and HSP27 in early detection HCC and to investigate their relation to clinicopathologic parameters of disease progression.

2. Patients and methods:

The current study was conducted on 76 patients (51 males and 25 females, age range 46- 71 years), 42 of them have HCC and 34 patients with liver cirrhosis (LC) without HCC. They attend outpatient and inpatient clinics of the Hepatology Department-National Liver Institute- Menoufiya University, and Tropical Medicine Department of Al Zahraa and National Cancer Institute from July 2010 till April 2012.

The HCC group includes: 30 males and 12 females with age range 51-71 years, diagnosed according to clinical examination, laboratory and radiological investigations including abdominal ultrasonography and triphasic C.T. abdomen. The group of LC were 21 males and 13 females with age range 46-66 years, they were selected according to clinical examination, abdominal ultrasonography, laboratory investigations and liver biopsy findings.

Exclusion criteria for patients: Any patients with cancer other than HCC, septicaemia, chronic inflammatory disorders, chronic heart disease were excluded. None of the selected patients had received local or systemic therapy for HCC before.

Local ethics committee approval and informed consent from all participants in this study were obtained prior to testing.

Laboratory investigations:

Serum levels of AST, ALT, albumin, total and direct bilirubin, alkaline phosphatase and gamma glutamyle transpeptidase (GGT) were done using Integra-400 (Roche-Germany). Prothrombin concentration was done by Fibrintimer (Roche-Germany). Complete blood counts were measured by Sysmix K-21 automatic cell counter (Japan). AFP serum level was measured by an automated Eleceyes (Roche-Germany). HBV markers and HCV antibodies were assayed by EIA (COBAS-Amplicore, Germany). HCV-RNA levels were analyzed by real time polymerase chain reaction using a commercial kit (Roche Diagnostic, Branchburg, NJ) according to the manufacturer's instructions.

Sampling and *Real-Time Quantitative RT-PCR Analysis of HSPs*

Real-time PCR was performed using noncancerous and cancerous liver biopsies taken from patients who underwent surgical liver resection. Part of liver sample was stored in liquid nitrogen immediately after the operation and kept at -80ºC until RNA extraction and the other part was fixed in 10% neutral buffered formalin and embedded in paraffin for histological studies.

Total ribonucleic acid (RNA) was isolated from liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the protocol recommended by the manufacturer. Reverse transcription reactions were performed using a Rever Tra Ace alpha-First Strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). Briefly, 1 μg of total RNA, 1 μL of oligo dT-primer, and 2 μL of dNTPs were incubated at 65°C for 5 min, then 10 μL of a cDNA synthesis mixture was added and this mixture was incubated at 50°C for 50 min. The reaction was terminated by adding 1 μL of RNaseH and incubating the mixture at 37°C for 20 min., all RNA samples were treated with DNase I to remove genomic DNA.

Real-time quantification of HSPs mRNA transcripts was performed as reported previously [26].

The PCR reaction was carried out in a tube containing a mixture of 2 μL cDNA, 12.5 μL 2 X SYBR Green (Applied Biosystems), 0.5 μL of 25 nM sense and antisense primers, and H2O up to final volume of 25 μL. For standardization of the amount of RNA, expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample was quantified by using GAPDH primers.

The primer sets used for HSPs and GAPDH are the following:

HSP70:	5′-AGGCCGACAAGAAGAAGGTGCT-3′ (forward) 5′-TGGTACAGTCCGCTGATGATGG-3′ (reverse)
HSP27:	5′-CCCACCCTCTATCACGGCTAC-3′ (forward) 5′-GGGCTCAACTCTGGCTATCTC-3′ (reverse)
GAPDH:	5′-GAAGGTGAAGGTCGGAGTC- 3′ (forward) 5′-CCCGAATCACATTCTCCAAGAA-3′ (reverse)

The PCR conditions were 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Real-time detection of the emission intensity of SYBR Green was performed with an ABI prism 7700 Sequence Detector (Perkin-Elmer Applied Biosystems), as reported previously **[27].** Multiple negative water blanks (no template control) were included in every analysis. Samples were also tested to ensure that they were negative for DNA using a complete master mix without reverse transcriptase (no amplification control). No amplification was observed for these controls, indicating the specificity of the assays for the respective mRNAs.

Statistical analysis:

Data were coded and summarized using SPSS (statistical package for Social Sciences) version 13.0 for Windows. Quantitative variables were described using mean ± standard deviation and categorical data by using frequency and percentage. Comparison between groups was done using Chi square (X^2) test for qualitative variables, student t test for normally distributed variables and Mann Whitney (U) test for none normally distributed quantitative variables. *P* value <0.05 was considered statistically significant.

3. Results:

The clinical, radiological and laboratory characteristics of the patients are demonstrated in table (I), the comparison between patient groups are shown in table (2).

Overexpression of HSP70 was detected in 39 (92.86%) out of 42 cases of HCC which is statistically significant compared to LC (p<0.001), in which one case only (2.94%) has overexpression out of 34 cases of LC. HSP27 was overexpressed in 3 cases (8.82%) of LC and in 24 (57.14%) out of 42 HCC, which is significantly higher (<0.01) than LC cases (Table 3).

In HCC cases, overexpression of HSP70 was associated with early HCC diagnostic parameters (tumor size, p<0.05) and prognostic criteria: vascular invasion (p<0.05), tumor grade (p<0.01), while not related to AFP levels or tumor number (Table 4).

Overexpression of HSP27 was associated significantly with (tumor size, p<0.05) and tumor number, and not associated with each of AFP, vascular invasion and tumor grade (p>0.05) (Table 5).

Table (1) Patients characteristics

Parameters	HCC patients (N=42)	LC patients (N=34)
Age (years) : (M±SD)	59.2±10.8	54.6±11.3
Gender:		
Male	30 (71.4%)	21 (61.8%)
Female	12 (28.6%)	13 (38.2%)
Mode of infection:		
Surgical procedures	24 (57.2%)	21 (%)
Blood transfusion	5 (11.9%)	2 (%)
Drug abuse	3 (7.1%)	4 (%)
Unknown causes	10 (23.8%)	7 (%)
Ultrasonographic Data:		
Hepatomegaly	31 (73.8%)	25 (73.5%)
Splenomegaly	27 (64.3%)	21 (61.8%)
Ascites	4 (9.5%)	2 (5.9%)
Hepatitis markers:		
HCV antibodies	36 (85.7%)	30 (88.2%)
HBS Ag	6 (14.3%)	4 (11.8%)
Child-Pugh classification:		
Class A	13 (31.0%)	14 (41.2%)
Class B	21 (50.0%)	15 (44.1%)
Class C	8 (19.0%)	5 (14.7%)

Table (2) Comparison between laboratory data of patient groups

Parameters	HCC patients (N=42) M±SD	LC patients (N=34) M±SD	Test of Sign.	*p*-value
ALT (U/L)	76.5± 21.4	68.7±18.2	1.97	>0.05
AST (U/L)	97.2±32.2	72.2±20.4	2.75	>0.05
GGT (U/L)	57.1 ±11.9	35.2±10.3	3.86	<0.05
ALP (U/L)	108.2±33.7	43.2±8.1	5.72	<0.01
T.bilirubin (mg/dl)	3.63 ±1.95	5.66 ±3.4	2.07	>0.05
S.albumin (g/dl)	2.98±0.35	3.01±0.68	1.13	>0.05
Proth.Con (%)	62.7± 10.2	58.6±9.7	1.84	>0.05
HB (g/dl)	11.4 ± 2.3	10.9 ± 1.6	1.93	>0.05
WBCs ($X10^9/L$)	8.6 ± 3.5	7.5± 2.8	2.01	>0.05
Platelets ($X10^9/L$)	132.6 ± 24.5	114.2 ± 15.2	2.65	>0.05
AFP (ng/ml)	408.5±367.5	18.43±7.41	6.73	<0.001

P<0.05 & <0.001 are statistically significant, p>0.05 is statistically non significant.

Table (3) Comparison between patient groups as regard HSP mRNA expressions.

Parameters	HCC patients (N=42)		LC patients (N=34)		X^2	*p*-value
	No	%	No	%		
HSP70 mRNA:						
Overexpression	39	92.86%	1	2.94%	12.73	<0.001
Minimal expression	3	7.14%	33	97.06%		
HSP27 mRNA:						
Overexpression	24	57.14%	3	8.82%	8.51	<0.01
Minimal expression	18	42.86%	31	91.18%		

P<0.01 & <0.001 are statistically significant**.**

Table (4) Comparison between HSP70 mRNA expression and prognostic data of HCC patients

Parameters	**HSP70 Overexpression** (N=39)		X^2	*P*-value
	No	**%**		
AFP levels:				
<100 ng/ml	18	46.15%	2.82	>0.05
≥100 ng/ml	21	53.85%)		
Tumor size:				
<5 cm	7	17.95%	4.16	<0.05
>5cm	32	82.05%		
Tumor number:				
Single	11	28.21%	2.01	>0.05
Multiple	28	71.79%		
Vascular invasion:				
Absent	6	15.38%	4.09	<0.05
Present	33	84.62%		
Tumor grade:				
G 1	2	5.13%	7.21	<0.01
G 2	9	23.08%		
G 3	28	71.79%		

P<0.05 & <0.01 are statistically significant, *p*>0.05 is statistically non significant.

Table (5) Comparison between HSP27 mRNA expression and prognostic data of HCC patients

Parameters	**HSP27 Overxpression (N=24)**		X^2	*P*-value
	No	**%**		
AFP levels:				
<100 ng/ml	11	45.83%	2.11	>0.05
≥100 ng/ml	13	54.17%		
Tumor size:				
<5 cm	5	20.83%	5.21	<0.05
>5cm	19	79.17%		
Tumor number:				
Single	8	33.33%	4.89	<0.05
Multiple	16	66.67%		
Vascular invasion:				
Absent	10	41.67%	1.59	>0.05
Present	14	58.33%		
Tumor grade:				
G 1	11	45.83%	2.75	>0.05
G 2	7	29.17%		
G 3	6	25.00%		

P<0.05 is statistically significant, *p*>0.05 is statistically non significant.

4. Discussion:

Heat-shock proteins have dual roles as a modifier to protein activities and as a central regulator in both cell proliferation and apoptosis **[27, 28]**. Among them, HSP70 and HSP27 are often overexpressed in cells of various cancers and have been suggested to contribute to tumorigenesis **[22, 29]**.

Previous studies have shown up-regulated expression of HSP70 and HSP27 in tumor cells, including those of HCC. HSP70 and HSP27 among HSPs are of special relevance in human cancer

inhibiting apoptosis **[22, 30]** .They were frequently stained in the cytoplasm and nuclei of tumor cells, but not in non-neoplastic hepatocytes **[30].**

Although early HCC is defined on the basis of histopathologic **[31]** and clinical studies **[32]**, the molecular changes that occur in early HCC are not well understood. Early HCC is characterized by an increase of cell density and growth **[33]**, but no positive molecular marker has yet been identified. Gene expression profiling is a promising method for finding molecular markers useful for the diagnosis of early cancer [26].

Our results revealed that, overexpression of HSP70 m RNA were detected in 92.86% of early HCC cases, while the expression was noticed in 2.94% of LC cases without HCC. Overexpression of HSP27 m RNA was detected in 57.14% of HCC cases and in 8.82% of LC cases. Several studies have evaluated expression of HSP70 in HCC **[22, 24-26 and 30]**. Consistent with our findings, all studies have reported overexpression of HSP70 in HCC tissues. **Chuma *et al.* [26]** revealed significant overexpression of HSP70 in early HCC compared with precancerous lesions.

Overexpression of HSP70 also has been observed in cultured cells transformed with c-myc or H-ras, or cotrans formed with H-ras/p53, suggesting involvement of HSP70 in transformation or progression **[34, 35]**., **Yokoyama *et al.* [36]** listed examples of HSP70 upregulation in human HCC tissues, and **Yin [34]**, and ***Chuma et al.*,[26]** described overexpression of HSP70 in HCC. It is possible that HSP70 expression increases as a result of tumorigenesis. For example, a stressful environment in early HCC (nutrient depletion and hypoxia resulting from insufficient blood supply **[32]**, may stimulate HSP synthesis.

Effendi & Sakamoto [37] detected immunoreactivity of HSP70 ranging up to 80% in most cases of early HCC, while no or only focal and faint nuclear staining was observed in the noncancerous background liver tissue. HSP70 is a housekeeping gene that assists with a variety of vital intracellular chaperoning functions. Expression of HSP70 increases under conditions of environmental cellular stress **[7]** and overexpression of HSP70 also leads to significant protection against cell apoptosis **[38]**. This is consistent with pathological processes such as oncogenesis. Although it is not yet clear, the stressful environment of early HCC (nutrition depletion, and hypoxia resulting from insufficient blood supply) may stimulate the expression of HSP70.

The roles of HSP70 in promoting tumor cell proliferation were previously observed in the various types of carcinoma **[33, 39]**, but not in HCC. Some studies, shown that HSP70 antisense oligomers can specifically inhibit tumor cell proliferation by inducing apoptosis **[40,41]**; these results suggest that anti-apoptotic functions of HSP70 may play an important role in tumor cell proliferation and tumor progression in HSP70 over-expressed tumors like HCC. Another plausible role for HSPs in tumorigenesis is as a modifier of protein activities **[42, 43]**. The tumor suppressor p53 protein, involved in the control of cell proliferation, represses transcription by direct protein-protein interaction with the promoter region of the human *HSP70* gene **[28]**.

Youshida *et al.* [25] study reported that immunohistochemically confirmed that HSP70 protein levels are significantly higher in progressed HCC than in early HCC and a significant association was found between immuno-reactivity for HSP70 and high histological grade of HCV-related HCC. HSP70 plays an important role as a chaperone of intracellular peptide antigens in cancer immunotherapy.

Chuma *et al.* [26] further showed that HSP70 could be a sensitive marker for the differential diagnosis of early HCC from precancerous lesions or noncancerous liver tissue, a diagnosis that is difficult even for pathologists because of the very well differentiated histology with minimum atypia seen in early HCC. Indeed, HSP70-p53 complexes can be detected in extracts from human cancer tissues **[22]**. **Cui *et al.* [44]** has reported that coexpression of anti-HSP70 and anti-p53 was shown in 3 out of 12 HCC tissues (25%). Therefore studying the coordinated expression of HSPs with p53 protein may be helpful to understand the roles of HSPs in the regulation of p53 function.

As an apoptosis inhibitor, HSP is overexpressed in human HCC tissues and correlated with carcinogenesis, progression and prognosis of HCC. Enhancement of intracellular HSP is closely related to the formation and development of HCC and a vital marker indicating the progression and aggravation of HCC **[45]**.

In the current study, HSP70 overexpression was specifically detected in HCC cases and showed a close relationship to the pathologic parameters related to tumor progression, such as large tumor size, portal vein invasion, and high tumor grade, **Joo *et al.* [22]** reported the same results as regard HSP70. Also, **Youshida *et al.* [25]** found that HSP70 expression may be related to tumor differentiation and tumor growth in HCV-related HCC. **Yao *et al.* [30]** added that, HSP70 was correlated with high Ki-67 labeling indices, large tumor size, presence of portal vein invasion, and high tumor stage. HSP27 was significantly related to the subgroup of HBV-associated HCCs, but not to others.

Our results reported that, the overexpression of HSP27 was associated significantly with tumor size and tumor number, and not associated with each of AFP, vascular invasion and tumor grade. **Seung *et al.* [46]** reported that HSP27 expression was related to histologic grade and the survival of patients with HCC. While, **Harimoto *et al.* [47]** found that, the HSP27 expression did not correlated with clinicopathologic factor in the overall group of HCCs. Also, **Joo *et al.* [22]** were not able to find any correlation between HSP27 immuno-reactivity and the other parameters examined.

Conclusion:

In conclusion, expressions of HSP70 and HSP27 may play an important role in hepatocarcinogenesis, and especially HSP70 showed a close relationship to the pathological parameters associated with tumor progression. Our results could be additional evidence that HSP70 expressions can contribute to not only hepatocarcinogenesis but also tumor progression, but also is expected to be an ideal target for the therapy of hepatocellular carcinoma. Nevertheless, further analysis is still necessary to carefully evaluate the roles of these molecular pathology candidates in early hepatocarcinogenesis.

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