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Overexpression of heat shock protein HSP90AA1 and translocase of the outer mitochondrial membrane TOM34 in HCV-induced hepatocellular carcinoma: A pilot study

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ABSTRACT

Objective: Identification of new molecular markers to enhance early diagnosis, prognosis and/or treatment of hepatocellular carcinoma (HCC) is a need. TOM34 (34 kDa-translocase of the outer mitochondrial membrane) protein expression deregulation has demonstrated to be involved in the growth of many cancers. Here, we aimed at evaluating serum *TOM34* and some heat shock proteins (*HSPA4*, *HSPA1B*, and *HSP90AA1*) expressions in hepatitis C virus (HCV)-related cirrhosis and HCV-induced HCC relative to controls and correlating these expressions to the clinicopathological data.

Methods: Serum specimens were collected from 90 patients with HCV associated complications (30 cirrhotic, 30 early HCC and 30 late HCC) and 60 controls. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed for relative quantification of the four target genes using the Livak method. In silico network analysis was also executed to explore the contribution of the genes in liver cancer.

Results: The serum *TOM34* and *HSP90AA1* transcripts were significantly upregulated in HCC patients compared to cirrhotic ones with more up-regulation in late HCC patients. Receiver operating characteristic analysis showed the optimum cutoff value of 0.625 corresponding to 71.7% sensitivity and 56.7% specificity, and an area under the curve (AUC) of 0.705 to discriminate HCC from cirrhotic groups (P = .002). In multivariate analysis, ordination plot showed obvious demarcation between the study groups caused by the higher levels of *TOM34* among other variables.

Conclusions: *TOM34* and its partner *HSP90AA1* might be used as a potential biomarker for monitoring HCV-induced HCC progression in the Egyptian population. Future large-scale validation studies are warranted.

1. Introduction

Hepatitis C virus (HCV) is a single stranded RNA virus that infects the human liver and causes a major global health concerns with a sustained increase in the morbidity and mortality [1]. In 2016, the prevalence of HCV was estimated to be 2.5% of the global population and about 7 million Egyptians, between 15 and 59 years old, had chronic HCV infection in 2015 [2]. The chronicity of HCV can lead to the development of liver cirrhosis (LC) after approximately 15–25 years of infection which may lead to hepatic decompensation, hepatocellular carcinoma (HCC), and death in a considerable proportion of patients [3,4].

Worldwide, HCC is the second most common cause of cancer and the most common in Egypt [5]. With regard to patients with chronic HCV infection, the annual incidence rate of HCC in cirrhotic patients is estimated to be 3%–5% per year [7]. Genetic changes, in either the

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gene sequence or expression, are a well-established risk factor for the initiation and progression of tumor cell. A growing body of evidence has linked the development of HCC with a number of gene mutations, activation of oncogenes, and up-regulation of certain gene profile [8,9]. Moreover, Kim and Park [9] found distinguished differences in gene expression patterns between normal liver, LC, and HCC, which reflects the importance of identification of the expression profile of potentially affected genes to explore their diagnostic and prognostic values.

Emerging studies suggested the role of molecular chaperones in proteostasis maintenance in normal and cancer status [10,11]. Molecular chaperons are helper proteins that assist in folding and/or assembly of newly synthesized and existing large peptide chains to prevent their aggregation into non-functional tertiary structures, hence known as guardians of the proteome [11]. Of the 332 known human chaperones, 147 genes encode for heat shock proteins (hsp) and are endogenously expressed in response to cellular stresses as hyperthermia, hypoxia, oxidative stress, and toxin exposure [12]. They are classified according to molecular weight of their monomers (Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and small Hsp families). They prevent or correct any damage caused by protein misfolding during biological processes and they are expressed prior to the induction of target proteins to maintain spatial and temporal regulation of protein synthesis [12]. Many proteins require more than one chaperone and ATP hydrolysis for correct folding. Chaperones adopt foldase or holdase type of activity. The former (e.g. Hsp70) bind partially or totally misfolded proteins, unfold and correctly refold them, while the later chaperones (e.g. Hsp90) hold aggregation-prone partially misfolded substrates prevent their aggregation and stabilize them [10]. Cells can regulate the duration and strength of Hsp activity via tight auto-regulatory mechanisms [12].

Failure of molecular chaperone network is a hallmark of various human diseases. Misfolded or disaggregated proteins increased the risk of formation of toxic oligomers and non-functional protein copies which could also recruit wild-type protein forms. Cancer cells were found to exhibit altered levels of chaperons [13]. In addition, rapidly growing cells acquire genomic instability properties which disturb cellular proteostasis through the accumulation of mutant forms of unstable proteins with further overwhelming of negative feedback loop of chaperones. Disease-related mutations in Hsp family members have been identified [12]. Hsp90α which is coded by *HSP90AA1* gene is the major cytosolic chaperon in eukaryotes; it works on cytoprotection and intracellular signaling, controls protein homeostasis folding and assembles of secretary polypeptides in endoplasmic reticulum (ER), and modulates post-translational translocation of proteins across the membranes of organelles [14]. While Hsp70 family which includes Hsp70-4 coded by human *HSPA4* gene, and Hsp70-hom coded by human *HSPA1B* gene, is ubiquitous principal folding chaperone, responsible for folding de novo proteins and their transport into ER and mitochondria, and promotes lysosomal degradation of cytosolic proteins [10]. Hsp90 and Hsp70 together modulate the late-stage folding and maturation of proteins [11], including transcription factors, kinases, E3 ubiquitin ligases, signaling and tumor promoting proteins [15], thus shedding the importance of multiprotein hsp90/hsp70 chaperone machinery as promising drug targets [16].

A number of proteins located in the mitochondrial membrane are essential for recognition and translocation of some precursor proteins into the mitochondria [17]. The translocase of the outer mitochondrial membrane (TOM) is considered the main protein entry gate of mitochondria [18]. Among them, TOM34 represents a scaffolding co-chaperone of HSP90/HSP70 complex during protein folding [19]. While mitochondrial dysfunction is a consistent feature of cancerous cells, previous reports demonstrated a statistically significant up-regulation of *TOM34* and its encoding protein in a number of cancers including colorectal cancer (CRC) [20], breast cancer [17], and lung cancer [21]. The over-expression of *TOM34* profile in cancer cells may explain the different mechanisms of acquired resistance to targeted therapy [21]; it can contribute for early detection of cancer growth and development of novel anticancer drugs as well [20].

Taken together, in the present study, we aimed to explore the expression profile of four genes (*TOM34*, *HSPA4*, *HSPA1B*, and *HSP90AA1*) in sera of HCV-induced HCC patients compared to normal and HCV-associated chronic liver disease patients, their correlations with clinicopathological data, and their potential utility as diagnostic or prognostic biomarkers.

2. Subjects and methods

2.1. Study participants

Ninety consecutive HCV-associated chronic liver disease patients and 60 age- and sex- matched healthy controls recruited from blood bank donors were enrolled in the study. Patient group included 30 individuals with hepatitis C-related LC, 30 cirrhotic- and HCV-induced early HCC, and another 30 cirrhotic- and HCV-induced late HCC. Patients had no evidence of metabolic or autoimmune liver disease, according to the clinical history and appropriate laboratory test results retrieved from their medical reports. Patients were recruited from the General Surgery and Oncology Clinics of the Suez Canal University (SCU) Hospital and from the Gasteroenterology Endoscopic ward of the Assuit University Hospital, Egypt, during the period between November 2014 and July 2015. Patients underwent "(1) thorough clinical assessment, (2) abdominal Ultrasonography and abdominal triphasic CT scan to confirm the presence of LC, portal hypertension, hepatosplenomegaly, HCC, and the presence of ascites, (3) quantitative viral load (HCV RNA) assessment by real-time TaqMan One-Step RT-PCR assay Reagents (Thermo Fisher Scientific, Waltham, MA, USA), and (4) prognostic scoring of liver disease by Child-Turcotte-Pugh classification". The Barcelona-Clinic Liver Cancer (BCLC) staging was used to assign patients into early and late HCC groups [22]. Control subjects were negative for anti-HCV antibodies, hepatitis B surface antigen, hepatitis B core antibodies, and human immunodeficiency virus antibodies with normal levels of liver enzymes. They had negative serum HCV RNA as detected by quantitative real-time PCR. Written informed consent was obtained from all participants. The study was conducted in accordance with the guidelines in the Declaration of Helsinki and approved by the Medical Research Ethics Committee of Faculty of Medicine, SCU (Approval No. 3137).

2.2. Specimen collection and the laboratory investigations

Fasting venous blood samples were collected from all participants for routine work-up, including fasting blood glucose, liver function tests, serum alpha feto-protein, complete blood count, and prothrombin concentration using commercially available assays as described in details in our previous work [23].

2.3. RNA extraction and reverse transcription

Serum total RNA was extracted by Qiagen miRNeasy Serum/Plasma Kit (Qiagen, Cat. No. 217184), following the manufacturer's protocol. All Samples were subjected to treatment with RNase-free DNase I (Qiagen, Hilden, Germany) for 2 h at 37 °C. RNA concentration and purity were determined by NanoDrop ND-1000 spectrophotometer (NanoDrop Tech., Inc. Wilmington, DE, USA) followed by agarose gel electrophoresis check for RNA integrity.

For reverse transcription (RT) reaction, High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used. For each total (20 μL) reaction, "RNA sample (10 ng) was added to RT 2× reaction mix (10 μL) containing 10× RT Buffer (2 μL), 25× dNTP Mix (100 mM; 0.8 μL), 10× RT random primers (2 μL), MultiScribe™ Reverse Transcriptase (1 μL), RNase inhibitor (1 μL), and nuclease-free water (3.2 μL)". RT was carried out in a

Table 1
Demographic and biochemical characteristics of the study groups.

Variables	Normal	LC	Early HCC	Late HCC	P values
Number	60	30	30	30	
Age					
Age, mean (SD)	48.8 ± 15.7	53.4 ± 7.24	50.7 ± 9.2	55.2 ± 7.2	0.074
Age groups (year)					
< 55 y	34 (567)	16 (53.3)	21 (70.0)	12 (40.0)	0.135
≥55 y	26 (43.3)	14 (46.7)	9 (30.0)	18 (60.0)	
Sex					
Females	14 (23.3)	7 (23.3)	6 (20.0)	4 (13.3)	0.710
Males	46 (76.7)	23 (76.7)	24 (80.0)	26 (86.7)	
Family history	–	24 (80.0)	1 (3.3)	17 (56.7)	< 0.001
Laboratory data					
ALT (U/L)	55.6 ± 19.5	105.3 ± 62.7[a]	93.2 ± 48.3[a]	95.8 ± 100.3[a]	< 0.001
AST (U/L)	50.1 ± 22.7	81.9 ± 62.5[a]	80.5 ± 37.1[a]	75.6 ± 49.7[a]	0.001
ALP (U/L)	131.9 ± 18.4	129.1 ± 50.4	151.3 ± 39.11	137.8 ± 30.2	0.041
Bilirubin (mg/dL)	0.54 ± 0.1	3.6 ± 3.3[a]	3.1 ± 2.6[a]	4.8 ± 3.7[a]	< 0.001
Albumin (g/L)	36.1 ± 7.8	23.2 ± 8.7[a]	26.8 ± 4.5[a]	24.4 ± 5.5[a]	< 0.001
FBS (mmol/L)	7.7 ± 1.6	7.8 ± 4.2	9.4 ± 4.3	11.1 ± 4.2[a]	< 0.001
AFP (μg/L)	–	31.1 ± 45.6	689.7 ± 283	730.9 ± 794.3	< 0.001
HB (g/dL)	11.2 ± 1.5	9.04 ± 3.1[a]	10.09 ± 1.5	9.7 ± 1.7[a]	< 0.001
WBC ($\times 10^9$/L)	5.23 ± 1.8	5.45 ± 4.2	4.9 ± 2.3	8.8 ± 10.1[a]	0.010
PLT ($\times 10^9$ /L)	134.8 ± 37.6	105.7 ± 46.1[a]	95.2 ± 14.7[a]	95.7 ± 36.3[a]	< 0.001
PT (sec)	12.7 ± 1.9	17.9 ± 7.8[a]	15.0 ± 2.75	17.5 ± 5.5	< 0.001

Data are shown as number (percentage) or mean ± SD. LC: liver cirrhosis; HCC, hepatocellular carcinoma patients; CTP, Child-Turcotte-Pugh classification for liver cell failure; tumor staging by BCLC, Barcelona-Clinic Liver Cancer staging system for HCC patients; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; PC, prothrombin concentration; PT, prothrombin time; AFP, alpha fetoprotein; HB, hemoglobin count; WBC, while blood cells; PLT, platelet count. Chi-square test was used for qualitative variables and ANOVA test was used for quantitative variables followed by multiple comparison Tukey HSD test.
[a] Compared to normal. P value < .05 was considered as statistically significant.

Mastercycler Gradient Thermocycler (Eppendorf, Hamburg, Germany) at 25 °C for 10 min, followed by 37 °C for 120 min, and finally 85 °C for 5 min, then hold at 4 °C. Appropriate negative controls were included in each run [23].

2.4. Quantitative reverse transcriptase-polymerase chain reaction

Real-time PCR assays were performed in accordance with the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines" [24]. The relative expression of the four study genes were quantified using TaqMan® assays (Applied Biosystems, assay ID Hs00199069_m1 for *TOMM34*, Hs04977836_g1 for *HSPA4*, Hs01040501_sH for *HSPA1B* and Hs05050555_s1 for *HSP90AA1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) endogenous control assay (TaqMan®, Thermo Scientific, catalogue no 4331182), and Universal PCR master mix II, No UNG (2×) (Taqman®, Applied Biosystems, P/N 4440043). All reactions were run in duplicate and included a no-template control (with water instead of cDNA), and a no-RT control. PCRs were carried out in a total volume of 20 μL using StepOne™ Real-Time PCR System (Applied Biosystems) as described in details previously [25]. Each 96-well plate run initially for 5 min at 95 °C, followed by denaturation for 15 s at 95 °C, annealing for 1 min at 60 °C, and elongation for 1 min at 72 °C, repeated 40 cycles.

2.5. Multivariate analysis

Data was examined for outliers and no transformation was required. Ordination analysis was carried out using Non-metric multidimensional scaling (NMS) ordination technique to visualize gene expression profile of patients along axes according to their resemblances. NMS was adjusted to attempt 50 times with Sorensen coefficient as a distance measure.

2.6. In silico functional network analysis of genes

COMPARTMENTS database was used (compartments.jensenlab.org/) to identify the subcellular localization of chaperon proteins. Protein-protein network was carried out using STRING database version 10.5 (string-db.org) based on physical interactions and functional associations. Functional enrichment analysis for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and gene ontology terms of the studied genes was done.

2.7. Statistical analysis

Statistical Package for the Social Sciences (SPSS) for Windows software (version 20.0) was used for data processing. The software package named PC-ORD ver. 5 [26] was employed for multivariate analysis. Our calculations by G power-3 showed that with the specified study design (gene expression), and allowable error rates; alpha error = 0.5 with sample size at least 21 for each group can give 80% power with a large effect size (http://www.gpower.hhu.de/).

Comparison of categorical variables was done, using chi-square (χ^2) or Fisher's exact tests where appropriate. While analysis of variance in case of normally distributed data, Mann-Whitney U (MW) and Kruskal-Wallis (KW) tests on skewed data, were used to compare continuous variables, followed by appropriate multiple comparison tests. The statistical significance was considered at p-value (two-tailed) < 0.05. The fold change of mRNA expression in each patient cancer tissue relative to the mean of controls was calculated using Livak method based on the quantitative cycle ($C_q = C_T$) value with the following equation: relative quantity = $2^{-\Delta\Delta CT}$ method [27].

3. Results

3.1. Baseline characteristics of the study populations

The mean age of the included participants was 55.2 ± 7.2 in late HCC group, 50.7 ± 9.2 in early HCC group, 53.4 ± 7.24 in LC group, and 48.8 ± 15.7 in healthy volunteers group; with no statistically significant difference between groups (p = .07). The number of the included participants that presented with fatigue, lipido, anorexia, jaundice, lower limb edema, and bleeding was significantly higher among HCC group compared to LC group (p < .05). Patients with early

Table 2
Comparison of gene expression in studied groups stratified by age.

	Age							
	< 55 years				55 years and more			
	LC	Early HCC	Late HCC	P value	LC	Early HCC	Late HCC	P value
Total No	16	21	12		14	9	18	
TOM34	0.33(0–2)	1.54(0–28)	1.37(1–69)[a]	0.009	1(0–7)	2.92(0–8)	7.93(0–347)[a]	0.060
HSPA4	22.9(0–438)	80.1(0–752)	127(0–207)	0.400	33.5(0 − 321)	43.0(0–143)	97.3(2–574)[a]	0.058
HSPA1B	0.8(0.17–12)	0.8(0.1–86)	1.4(0.2–30)	0.300	0.7(0.16–13)	2.0(0.1–7.2)	3.7(0.28–38)	0.300
HSP90AA1	0.8(0–56)	0.6(0–34)	1.0(0–36)	0.500	1.05 (0–87)	1.27(0–3)	5.8(0–143)[a,b]	0.005

Data are presented as median (quartiles). LC: liver cirrhosis, HCC: hepatocellular carcinoma patients, cancer stage in HCC patients according to Barcelona-Clinic Liver Cancer staging system. Kruskal-Wallis test was applied, followed by Dunn-Bonferroni post hoc test. P < .05 is significant.
[a] Statistically significant compared to LC group.
[b] Statistically significant compared to the early HCC group.

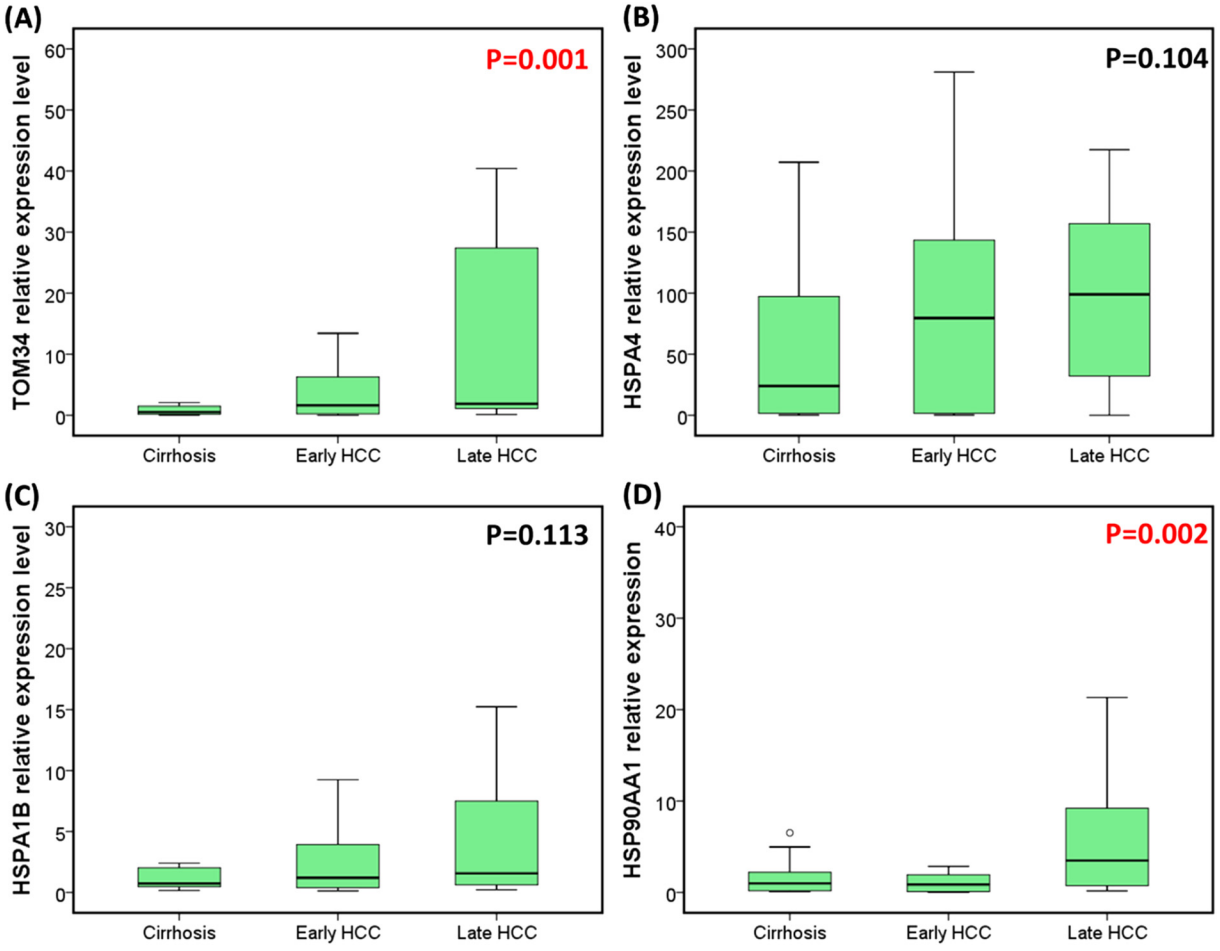

Fig. 1. Gene expression of chaperon and mitochondrial proteins among the study population. Values are presented as medians. The box defines upper and lower quartiles (25% and 75%, respectively) and the whiskers indicate upper and lower adjacent limits. Fold-change for each gene was normalized to *GAPDH* and calculated using delta-delta CT method ($=2^{(-\Delta\Delta CT)}$) compared to healthy controls. Relative expression level of control group was set at the value of 1. HCC; hepatocellular carcinoma. The Kruskal Wallis test was used.

HCC showed a statistically significant higher SBP (148.0 ± 19.7) and temperature (37.5 ± 0.47) compared to LC and healthy volunteer groups ($p < .005$). The majority of HCC patients (65%) were in Child C category compared to only 23% of LC patients. As expected, HCC patients showed a statistically significant more deterioration in physical examination and laboratory investigations compared to LC patients and healthy volunteers. Table 1 and Table S1 show the demographic, clinical, and biochemical characteristics of the study groups.

Furthermore, we performed a stratified analysis to show the gene expression among different age groups. The expression of *TOM34* remained significantly higher among late HCC patients who were < 55 years old [median 1.37 (1–69); $p = .009$]. While, the expression of *HSP90AA1* was only significantly higher among late HCC patients older than 55 years old [median 5.8 (0–143), $p = .005$] (Table 2). No association was found between the expression levels of the studied genes and CTP class of patients (all $p > .05$).

Table 3
Diagnostic performance of the studied genes.

	AUC	SE	95% CI	P value	Sensitivity	Specificity	Cutoff value
HCC versus LC							
TOM34	0.705	0054	0.599–0.811	0.002	71.7	56.7	0.625
HSPA4	0.630	0.061	0.509–0.750	0.046	63.3	64.3	49.09
HSPA1B	0.603	0.061	0.483–0.723	0.112	56.7	64.3	1.130
HSP90AA1	0.573	0.063	0.451–0.696	0.260	56.7	57.7	1.290
Late HCC versus early HCC							
TOM34	0.649	0.071	0.509–0.789	0.048	53.3	56.7	1.795
HSPA4	0.546	0.076	0.397–0.695	0.540	60.0	56.7	87.40
HSPA1B	0.599	0.074	0.454–0.744	0.188	60.0	53.3	1.275
HSP90AA1	0.740	0.065	0.613–0.867	0.001	73.3	60.0	1.265
Early HCC versus LC							
TOM34	0.619	0.075	0.471–0.766	0.114	60.0	60.0	0.800
HSPA4	0.594	0.076	0.446–0.743	0.209	63.3	63.3	49.09
HSPA1B	0.549	0.076	0.401–0.698	0.511	53.3	66.7	1.240
HSP90AA1	0.434	0075	0.287–0.580	0.379	50.0	50.0	0.945

ROC curve analysis was applied to discriminate between LC: liver cirrhosis, HCC: hepatocellular carcinoma patients; cancer stage in HCC patients according to Barcelona-Clinic Liver Cancer staging system. AUC: Area Under the Curve, SE: standard error, CI: Confidence Interval. P < .05 is significant.

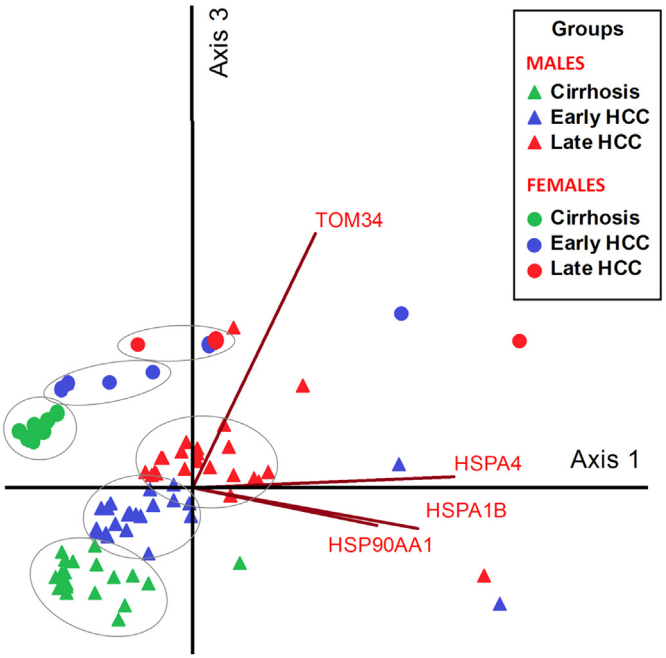

Fig. 2. Multivariate analysis by nonmetric multidimensional scaling (NMS) ordination analysis in patients. Non-metric multidimensional scaling (NMS) ordination technique was run to visualize gene data of patients along axes according to their resemblances. NMS was adjusted to attempt 50 times with Sorensen coefficient as a distance measure. Different genes' direction and power of correlation with axis 1 and 3 are demonstrated. Stratification analysis was done by gender and disease groups.

3.2. Expression profiling of chaperon and mitochondrial proteins

The median expression of *TOM34* in late HCC patients was 1.89 (0–347) which was significantly higher than early HCC patients (median 1.74 (0–28)) and LC patients (median 0.53 (0–7); $p = .001$). Similarly, the median *HSP90AA1* expression in late HCC patients (3.31 (0–143)) was significantly higher than early HCC patients (median 1.07 (0–34)) and LC patients (median 1 (0–87); $p = .002$). However, the expression of *HSPA4* and *HSPA1B* did not differ significantly between study groups (Fig. 1).

3.3. Diagnostic performance of chaperon and mitochondrial proteins

The ROC analysis indicated that the expression of *TOM34* and *HSPA4* genes had the ability to discriminate between cirrhotic and HCC patients (AUC = 0.705, p = .002; and AUC = 0.630, $p = .046$; respectively), at the cutoff values were 0.625 and 49.09, respectively. While the expression of TOM34 and HSP90AA1 gene could differentiate between early and late HCC patients (AUC = 0.649, $p = .048$ and AUC = 0.740, $p = .001$, respectively) with a cutoff value of 1.795 for TOM34 and 1.265 for HSP90AA1. Table 3 shows the diagnostic performances of all studied genes.

3.4. Multivariate analysis

Multivariate analysis using non-metric multidimensional scaling (NMS) (Fig. 2) demonstrated a distinctive separation of the gene expression values between male and female patients. Within each gender, the NMS joint plot shows the separation of three distinct groups of patients along axes 1 and 3 based upon the data collected from gene expression. Different genes' direction and power of correlation with axis 1 and 3 are demonstrated. TOM34 might be considered as potential gene responsible for the patient groups' separation. *TOM34* expression increased toward the patients with late hepatic carcinoma and less expression was shown with LC and early cancer patients. However, *HSPA4* expression values influenced a cluster of male patients with late HCC along axis 1. Lower values of *HSPA1B* and *HSP90AA1* expression caused clear discrimination between male and female patients.

3.5. Functional enrichment analysis

Subcellular localization of the proteins coded by the studied genes is depicted in Fig. 3 A–D. TOM34, HSPA4, HSPA1A/B, and HSP90AA1 are highly abundant in the nucleus and cytoplasm. TOM34 protein is existed in the mitochondrial outer membrane and integral component of membranes. Other HSPs are present in the endoplasmic reticulum, extracellular region, and mitochondria. HSP90AA1 is also found in lysosomes.

String analysis revealed binding relationships between HSP90AA1 and several partners, including TOM34, HSPA4, and HSPA1A/B. In addition, same chaperone elicited two other types of actions (activation and expression with inhibition) with HSPA1A/B and HSPA4, Fig. 3. From the network, HSP90AA1 seemed to have the driving influence and functional coordination with the studied molecular chaperone network; showing diverse interactions with various co-chaperones that modulate its substrate recognition and chaperone activity. It mainly functions in promoting the maturation, structural maintenance and proper regulation of specific target proteins involved in cell cycle control and signal transduction.

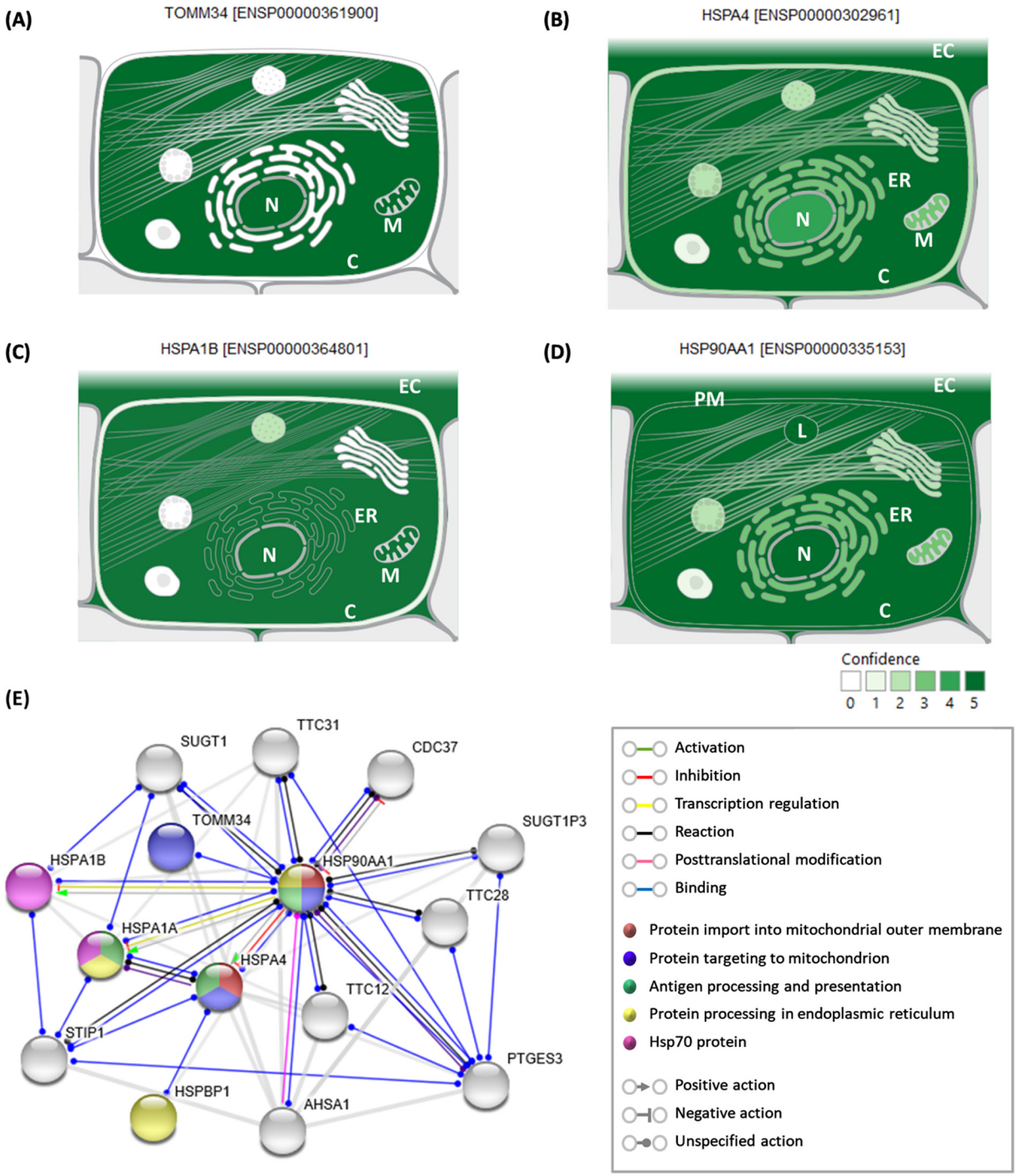

Fig. 3. Functional enrichment analysis of chaperon and mitochondrial protein.
(A–D) Subcellular localization of TOM34, HSPA4, HSPA1B, and HSP90AA1. Data source: Compartment database (https://compartments.jensenlab.org/). N: nucleus, C: cytosol, M: mitochondria, ER: endoplasmic reticulum, EC: extracellular region, PM: plasma membrane, L: lysosome. (E) STRING analysis. The network consists of 15 nodes (proteins) and 47 edges with protein-protein interaction enrichment *P* value of 6.66e-16 and minimum interaction requirement score at 0.7. The line shape indicates the predicted mode of action. STRING version 10.5 was used. TOMM34: Translocase of outer mitochondrial membrane 34, HSPA4: Heat shock 70 kDa protein 4, HSPA1B: Heat shock 70 kDa protein 1B, HSPA1A: Heat shock 70 kDa protein 1A, HSP90AA1: Heat shock protein 90 kDa alpha (cytosolic), class A member 1, HSPBP1: HSPA (heat shock 70 kDa) binding protein, cytoplasmic cochaperone 1, SUGT1: SGT1, suppressor of G2 allele of SKP1, CDC37: Cell division cycle 37 homolog, PTGES3: Prostaglandin E synthase 3 (cytosolic), STIP1: Stress-induced-phosphoprotein 1, AHSA1: AHA1, activator of heat shock 90 kDa protein ATPase homolog 1, TTC28: Tetratricopeptide repeat domain 28, SUGT1P3: Suppressor of G2 allele of SKP1, TTC12: Tetratricopeptide repeat domain 12, TTC31: Tetratricopeptide repeat domain 31.

4. Discussion

In the present study, we compared the expression profile of four genes (*TOM34*, *HSPA4*, *HSPA1B*, and *HSP90AA1*) between volunteers with normal liver, patients with LC, and patients with HCC. Our results indicated a statistically significant higher expression of *TOM34* gene, along with its partner *HSP90AA1*, in late HCC patients compared to cirrhotic and early HCC patients. While the expression of *HSPA4* and

HSPA1B genes were incomparable between the three groups. Our stratified analysis showed that the expression of *TOM34* and *HSP90AA1* remain significantly higher in late HCC male patients, regardless of age group, compared to healthy volunteers and cirrhotic patients. But the expression levels of both genes were comparable between the three groups among females. In addition, our ROC analysis indicated that the expression of *TOM34* and *HSPA4* genes had the ability to discriminate between cirrhotic and HCC patients, while the expression of *TOM34* and *HSP90AA1* genes could differentiate between early and late HCC patients.

To the best of our knowledge, this is the first study to measure the expression of *TOM34* and its chaperons in HCC patients. There is a growing body of evidence that demonstrated a significant association between the overexpression of *TOM34* and a number of cancers [17,20]. Moreover, a number of previous studies indicated a potential role of HSP family members in tumorigenesis and tumor prognosis [28,29]. Therefore, establishing a significant association between the overexpression of *TOM34* and the development of HCC in cirrhotic patients will help in the diagnosis and development of new targeted therapeutic agents.

Consistent with the role of *TOM34* upregulation in cancer, Aleskandarany and colleagues [17] found that *TOM34* overexpression was strongly associated with breast cancer distant metastases and poor prognosis in their study population. Furthermore, Shimokawa and colleagues [20] explored the relevance of *TOM34* expression in CRC. They found that TOM34 protein was mostly accumulated in CRC tissues in comparison to normal non-cancerous tissues. Interestingly, Shimokawa and colleagues were able to suppress cancer cells growth through transfecting HCT116 cells in the colon with short-interfering RNA (siRNA) targeting TOM34, which reflects the potential role of drugs targeting *TOM34* as a promising therapy. Similarly, a recent study reported that *TOM34* expression was found to be elevated in the majority of primary CRC (78%) and liver metastasis (73%) samples [30].

Our study has pointed out the importance of *TOM34* expression monitoring in the laboratory diagnosis of HCC. The expression of *TOM34* and *HSPA4* genes appeared to be sensitive and specific diagnostic biomarkers for discrimination between cirrhotic and HCC patients. Similarly, Shimokawa and colleagues [20] suggested that *TOM34* may contribute significantly for the diagnosis of CRC.

The association between the up-regulation of *HSP* and human malignancies appears to be well-established. *HSP70* and *HSP90* are often up-regulated in many cancers including lung cancer, gastric cancer, and breast cancer [31–33]. Our results showed a statistically significant higher expression of *HSP90AA1* in late HCC patients compared to early HCC patients. Similar to our results, a recent multicenter clinical trial showed that plasma Hsp90α concentrations were significantly higher in late HCC patients compared to early HCC patients or cirrhotic controls; however, plasma HSP90α did not differ according to the type or grade of liver cancer [34]. On the other hand, we could not detect a statistically significant difference in *HSP90AA1* gene between HCC patients and cirrhotic or healthy controls. In contrast, Gao and colleagues [35] reported that *HSP90* expression was higher in HCC cell lines compared to control group. Another report by Sun and colleagues [36] reported a significant overexpression of *HSP90* compared to control group. Leng and colleagues [37] reported that HSP90 could mediate HCC survival through regulation of survivin, cyclin D1, p53 and nuclear factor-κB. Therefore, HSP90 could represent a potential target in the HCC therapeutic regimen. AUY922, a novel HSP90 inhibitor, was reported to effectively inhibit the proliferation and migration of HCC cells [38]. The inhibition of HPS90 enhanced the efficacy of Bcl-2 inhibitors and rapamycin in HCC as well [39,40].

In the present study, we performed a string analysis to explore the potential interaction between the studied genes. There were significant interactions of *HSP90AA1* with *TOM34* and *HSPA4* in the experimental/biochemical data. Due to its tetratricopeptide repeat (TPR) domains, TOM34 is hypothesized to influence the HSP70/HSP90 interactions through binding to the EEVD-COOH motif [41]. Previous reports demonstrated that TOM34 may acts as co-chaperone of Hsp70 and Hsp90 facilitating their scaffolding and influence their interaction [19,42].

In conclusion, HCC was associated with a significant overexpression of *TOM34* and its partner *HSP90AA1*. *TOM34* monitoring could be a potential biomarker for early detection of HCC, while *HSP90AA1* could be a good discriminator for late HCC. As maintenance of cellular proteostasis capacity requires the coordination and interconnected interactions of the chaperone network, a controlled perturbation of the functional interaction between molecular chaperones and proteases could provide new avenues for therapeutic intervention. However one should consider the variability in the expression levels between male and female patients when setting the discriminative scale of diagnosis and prognosis.

Few limitations should be addressed; there was no proper documentation of the treatment regimens given to the patient and that prevented us from analysis the interaction of therapeutic response and gene expressions. Additionally, the sample size was relatively small, which might limit the statistical power and weaken potential associations. Hence, the role of *TOM34* overexpression should be examined in a larger cohort of patients and the interaction of different treatment strategies with the gene expression should be investigated. Furthermore, investigation of possible signaling pathway changes should be planned based on cellular and animal models, in order to explore the detailed biological mechanisms.

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Conflict of interest

The authors declare that they have no competing interests.

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