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Circulating Survivin and TIMP-1 in Hepatitis C Virus Associated Liver Fibrosis

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Authors' contributions

This work was carried out in collaboration between all authors. Author BEME managed the biochemical and molecular analyses of the study, wrote the first draft of manuscript and shared in the research design, statistical analyses and interpreted the results. Author AAAG shared in the research design as well as biochemical and molecular biology assays, statistical analyses and interpreted the results. Author SMAG wrote the protocol and managed the literature searches and shared in interpretation of results. Author SESB obtained the liver biopsies and blood samples and achieved the clinical investigations of the participants and shared in interpretation of results. Author MAAA shared in the research design, biochemical and molecular biology assays, statistical analyses, interpreted the results and revised the manuscript critically for important intellectual content. Author SRAR managed the literature searches and shared in samples collection, biochemical and laboratory works and interpreted the results. All authors edited the manuscript and approved its finally submitted version.

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ABSTRACT

Aims: The present study was conducted to assess the clinical utility of survivin, an anti-apoptotic protein, and the profibrogenic tissue inhibitor metalloproteinase-1 (TIMP-1) as non-invasive biomarkers for the discrimination between stages of HCV-associated liver fibrosis that are largely asymptomatic.
Methodology: Circulating survivin and TIMP-1 levels as well as their corresponding proteins and genes expressions were, respectively, assessed by ELISA, Western blot and RT-PCR in 100 HCV-infected patients at different stages of liver fibrosis in comparison to healthy controls.
Results: With the progression of hepatic fibrosis, each of circulating survivin and TIMP-1 as well as their ratio (survivin/TIMP-1) showed a stepwise increase and exhibited significant positive correlations with the fibrotic stage (r=0.98 & p=0.002, r=0.95 & p=0.01, and r=0.93 & p=0.013 respectively). A gradual increase in both survivin and TIMP-1 genes expression was also observed.
Conclusion: In conclusion, survivin, TIMP-1, and their ratio could represent promising biomarkers for prediction and discrimination of different stages of HCV-associated liver fibrosis. The higher sensitivity and specificity of survivin may provide new insights into its possible use as a target for the antifibrotic drugs. Further studies with validated tests on a large scale prospective clinical trial are required to ascertain these results.

Keywords: Survivin; tissue inhibitor of metalloproteinase-1; liver fibrosis; hepatitis C virus.

1. INTRODUCTION

Hepatitis C virus (HCV) infection is a universal health problem with an estimation of 130–170 million people infected throughout the world [1]. Globally, HCV infection shows a heterogeneous distribution with an average overall prevalence of 1–2% in most countries except in Egypt which is considered to have the highest worldwide prevalence (14.7% of the total population are seropositive for HCV, indicating that they had ever been exposed to the virus) [2-4]. More recently, in 2015 the Egypt Health Issues Survey (EHIS) reported that 6% of the Egyptians with age range of 1–59 years are seropositive for HCV. Of those individuals, 4% were found to have an active HCV infection [5].

The course of chronic HCV infection comprises long-term multistage processes including fibrosis that represents a dynamic scarring process associated with accumulation of collagen and extracellular matrix (ECM) proteins. This in turn, could lead to the distortion of the hepatic architecture by forming ECM complexes and a fibrous scar with potential progression to cirrhosis [6-8]. It was estimated that, in the majority of HCV-infected patients, progression to cirrhosis can occur asymptomatically after an interval of 15 to 20 years [6]. Therefore, there is an imperious medical need to regularly follow up the integrity of hepatic tissues in the susceptible patients.

Till recently, histological examination of the liver biopsy is considered as the gold-standard method for the assessment of fibrotic stage during the follow-up of patients [9,10]. Unfortunately, besides being an invasive procedure, the concomitant complications and the heterogeneous variations in the sampling process are considered as actual challenges for the widespread applicability of liver biopsy for accurate prediction of the progression of liver fibrosis [11]. Therefore, looking for reliable non-invasive methods for accurate assessments of different stages of liver fibrosis is a worthwhile investment.

It was reported that hepatic stellate cells (HSCs) which account for 5% to 8% of total liver cells are the major contributor of the fibrogenesis process [12]. In response to various stimuli from parenchymal injury, the accompanied inflammatory reaction generates large panels of profibrogenic signals that subsequently activate the quiescent HSCs to a myofibroblast-like phenotype. This in turn , may lead to the induction of ECM synthesis and deposition at the site of liver injury [13,14]. The balance between the ECM degrading matrix metalloproteinases (MMPs) and tissue inhibitors of the metalloproteinase family (TIMPs) was reported to be strongly regulated by HSCs [12]. Tissue inhibitor of metalloproteinase-1 (TIMP-1) is a 28 kDa protein that prevents collagen degradation through inactivation of MMPs, promoting liver fibrosis [15-17]. In order to play such a role during the liver fibrosis, HSCs are required to

exhibit an unusual high proliferation rate and enhanced survival via resistance of apoptosis [18]. Survivin, a 16.5 kDa member of the apoptosis inhibitors protein family, is up-regulated during HSCs activation, suggesting a potential role for survivin in protecting HSCs from apoptosis and regulating cell division and proliferation during hepatic fibrogenesis process [19,20].

Accordingly, the aim of the present study was to evaluate the utility of both survivin and TIMP-1 concentrations besides their ratio, genes and proteins expressions as reliable non-invasive tools for the prediction and discrimination of the different stages of HCV-associated liver fibrosis. Additionally, the current study aimed at investigating a possible correlation of those serum biomarkers with the clinical status of the HCV infected Egyptian patients.

2. PATIENTS AND METHODS

2.1 Study Population

The current study is a retrospective case-control study in which 100 chronic HCV-infected Egyptian patients from both sexes with their age ranging from 35 to 66 years were enrolled. They were randomely selected from a large cohert who were scheduled to receive antiviral therapy at Assiut Virology Unit for control of HCV from April 2014 to December 2014. Those patients were divided into five groups according to the histopathologically proven fibrotic stages of the needle liver biobsies by the METAVIR score [21]. The first group (F0 stage) included 20 patients without any degree of fibrosis. The second group (F1 stage) included 20 patients with portal fibrosis without septa. The third group (F2 stage) included 20 patients with portal fibrosis and few septa. The fourth group (F3 stage) included 20 patients with septal fibrosis without cirrhosis. The fifth group (F4 stage) included 20 patients with cirrhosis. F1, F2-F3, and F4 are considered as the mild, the moderate, and the advanced fibrotic stages respectively. The study incorporated also 20 age and sex matched hepatitis seronegative completely healthy subjects as a control group. All participants were subjected to thorough clinical investigations with a complete record of their detailed history.

The present study was approved by the Ethical Committee of the Faculty of Medicine, Assiut University, Egypt (IRB605/29/04/2014). An informed written consent was obtained from each participant.

2.2 Exclusion Creiteria

Patients were excluded if they had human immunodeficiency virus (HIV), hepatitis B virus (HBV), decompensated liver cirrhosis, comorbidities, schistosomiasis, chronic inflammatory liver diseases, hepatocellular carcinoma (HCC) associated cirrhosis or previously received interferon or any other antiviral therapy before enrolement in this study.

2.3 Blood Sampling

Eight ml venous fasting blood sample were collected from each subject at the time of registration for the study before any therapeutic measures under strict aspectic conditions and were delivered into 2 tubes: 4 ml into an EDTA treated tube for subsequent RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR) protocol and the other 4 ml were allowed to clot in another tube for serum isolation, which were kept frozen at -80 ^{o}C until used for laboratory estimations.

2.4 Biochemical Estimations

The criteria for HCV diagnosis are based serologically on detecting positive anti-HCV IgG, using enzyme linked fluorescent assay in human serum (VIDAS® bioMérieux SA, France). Subsequent quantitative determination of HCV RNA as an indicator of the viral load (IU/ml) in the HCV-IgG seropositive patients was established using a direct RT-PCR assay kit (Abbott GmbH, Germany). Quantitative estimations of serum survivin and TIMP-1 levels were achieved using the corresponding enzyme-linked immunosorbent assay (ELISA) kits (Biospes Co., Ltd., China). Serum albumin, total bilirubin levels as well as serum alanine aminotransferase (sALT) and serum aspartate aminotransferase (sAST) activities were estimated using corresponding commercially available colorimetric or kinetic assay kits.

2.5 Western Blotting Assessments of Survivin and TIMP-1 Proteins

Immunoblotting of both survivin and TIMP-1 proteins in serum samples was carried out using Western blot technique after the removal of the high abundant plasma proteins

(albumin and immmunoglobulines) using albumin and IgG Depletion SpinTrap kits (GE Healthcare Life Sciences, Sweden). Proteins in each corresponding serum sample were denatured at 95°C for 5 minutes in 2× Laemmeli buffer followed by addition of 5% 2-mercaptoethanol. SDS–PAGE electrophoresis was achieved by loading 30 µg protein per lane at 50 volts through stacking gel followed by 125 volts through 18% and 15% resolving gels for survivin and TIMP-1 respectively, during approximately 2 hours and transferred to a polyvinylidene difluoride (PVDF) membrane using T-77 Enhanced Chemiluminescence (ECL) semidry transfer unit (Amersham BioSciences, UK Ltd) for 2 hours. Immunoblotting was performed by incubating the PVDF membrane in a Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) and 5% non-fat milk for one hour at 4°C, followed by an overnight incubation at 4°C with rabbit anti-survivin polyclonal antibody (Bioss Inc., USA) or rabbit anti-TIMP-1 polyclonal antibody (Biospes Co., Ltd., China) at dilutions of 1:1500 and 1:1200, respectively. After being washed three times with TBST buffer, each membrane was incubated for one hour at room temperature with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Novus Biologicals, USA) at a dilution of 1:5000. Subsequent to four times washing with TBST, the membrane bound antibodies were detected with a commercially available BCIP/NBT substrate detection Kit (Genemed Biotechnologies, Inc., USA). Equivalent protein loading for each lane was confirmed by stripping and re-blotting each membrane at 4°C against mouse monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Novus Biologicals, USA) at a dilution of 1:1000. Each analysis was repeated to assure reproducibility of results. Quantification of each corresponding analysis was further performed using Image J software and expressed as the relative band density to the GAPDH.

2.6 RT-PCR Assessments of Survivin and TIMP-1 mRNA Levels

Total RNA was extracted from the whole blood using a total RNA purification Kit (Jena Bioscienc, Germany). The quality and quantity of the extracted RNA was controlled by a NanoDrop spectrophotometer (Biotech inc, USA). RNA quality was subsequently confirmed by gel electrophoresis. Then cDNA was synthesized from 2 µg total RNA using a RevertAid^{TM} First strand cDNA synthesis kit (Thermo Fisher Scientific Inc, USA). Quantification of the resultant cDNA was determined using the NanoDrop spectrophotometer. Amplifications of mRNA were performed by Biometra cycler (Jena, Germany) using a ready-to-use MyTaqTM mix kit (Bioline Inc., USA) and specific custom-made PCR primers (Vivantis Technologies Sdn. Bhd., Malaysia) to amplify the human survivin, TIMP-1 and β-actin-cDNA according to the NCBI reference sequences (Table 1). For survivin, samples were subjected to initial denaturation at 95°C for 3 minutes then underwent 35 cycles (denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 40 seconds) followed by a filling extension at 72°C for 5 minutes. For TIMP-1, the same procedures were used but with annealing temperature of 54°C for 30 seconds. RT-PCR of β-actin was performed in parallel as an internal control with annealing temperature of 58°C for 30 seconds. The RT-PCR products were analyzed by electrophoresis using 2% agarose gel (Roche Diagnostics, GmbH, Germany), stained with ethidium bromide and visualized by UV light using UV Transilluminator (UVT-20 M, Herolab, Germany). Each analysis was repeated to assure reproducibility of results. Quantification of each corresponding gel analysis was further performed using Image J software and expressed as the relative band density to the β-actin.

2.7 Statistical Analyses

Statistical analyses of the data were carried out using GraphPad prism (Graphpad Software, Inc., USA). Data comparisons were performed using analysis of variance (ANOVA) followed by Tukey's *t*-test. The levels of significance were accepted with $p<0.05$ and all relevant results were displayed as mean ± SD. The correlations between the continuous variables were performed using the Pearson's correlation. Sensitivity, specificity, the best cut-off values and the diagnostic efficacy index of survivin, TIMP-1 and survivin/TIMP-1 were calculated from the corresponding Receiver Operating Characteristic (ROC) curves.

3. RESULTS

3.1 Clinical, Demographic and Biochemical Data

Table 2 shows the clinical, demographic and biochemical data of the patients and controls.

Table 1. The primers used for amplification of survivin, TIMP-1 and β-actin in RT-PCR detections

Gene	Primer sequences (5'-3')	Product size (bp)	Annealing temp. (˚C)	Gen bank accession number
Survivin	F: AGTCCCTGGCTCCTCTACTG R: CGTGTGGAGAACGTGACAGA	380	55	U75285.1
TIMP-1	F: ACTTCCACAGGTCCCACAAC R: GCAGTTTGCAGGGGATGGAT	204	54	NM_003254.2
β-actin	F: AGCGGGAAATCGTGCGTGAC R: ACATCTGCTGGAAGGTGGAC	453	58	XM_006715764.1

3.2 Immunosorbent Assays of Circulating Survivin and TIMP-1

As shown in Table 2, serum survivin levels were significantly increased in F2, F3, and F4 groups in comparison to the healthy control group as well as the F0 and F1 fibrotic stages (p<0.001 for each). The increase in serum survivin levels was significant in F3 (p<0.05) and F4 (p<0.001) as compared to F2 fibrotic stage.

It is noticed that serum survivin levels showed a significant positive correlation with the fibrotic stage (r=0.98 & p=0.002, Fig. 1A, Table 3 and Table S1 in the supplementary data). Also, significant positive correlations were detected between serum survivin and each of sALT, sAST, total bilirubin and viral load (r= 0.64, 0.71, 0.44 and 0.49, respectively & p<0.0001 for each). While, a significant negative correlation was recorded between serum survivin and serum albumin level (r= –0.25 & p=0.004, Table 3 and Fig. S2 in the supplementary data).

On the other hand, serum TIMP-1 was significantly increased in the advanced stages of liver fibrosis (p<0.001 for each of F3 and F4) in comparison to the healthy controls. In comparison to F0, F1, and F2 fibrotic stages, the increase in serum TIMP-1 was significant in each of F3 and F4 (p<0.001 for each), as illustrated in Table 2.

Notably, serum TIMP-1 levels showed significant positive correlations with the fibrotic stage (r=0.95 & p=0.01), age (r=0.28 & p=0.0015), sALT (r=0.48 & p<0.0001), sAST (r=0.46 & p<0.0001), total bilirubin (r=0.33 & p=0.0002), and viral load (r=0.45 & p<0.0001) as illustrated in Fig. 1B and Table 3 as well as Fig. S3 and Table S1 in the supplementary data). Additionally, it exhibited an insignificant negative correlation with serum albumin (Table 3).

Interestingly, a strong positive correlation was detected between serum levels of survivin and TIMP-1 (r=0.51 & p<0.0001, Table 3 and Fig. S2 in the supplementary data). Moreover, the serum survivin/TIMP-1 ratio was significantly increased in the moderate (F2-F3) and advanced (F4) fibrotic stages in comparison to the healthy controls, non fibrotic (F0) and mild (F1) fibrotic stages (p<0.001 for each) as seen in Table 2.

The correlations between the fibrotic stage and the different parameters are represented in Table S1 and Fig. S1 in the supplementary data. Both age and sAST showed significant positive correlations with the fibrotic stage (r=0.98 & p=0.0016) and (r=0.95 & p=0.013) respectively. Also, sALT showed a positive correlation with the fibrotic stage but it did not reach the statistical significance (r=0.87 & p=0.051). Furthermore, there was a significant positive correlation between the fibrotic stage and survivin/TIMP-1 ratio (r=0.93 & p=0.019) as seen in Fig. 1C and Table S1 in the supplementary data.

3.3 ROC Analyses in Different Disease Stages

The overall diagnostic performance of each of serum survivin, TIMP-1 and their ratio in discriminating HCV fibrotic patients was assessed by ROC curve analysis (Fig. 1D-F). It is evident from the results that the best cut-off value of serum survivin was 1125 ng/L with 81% sensitivity and 95% specificity producing area under the curve (AUC) = 0.90 with AUC 95% confidence interval of 0.8468 to 0.9547 and *p<0.0001*. In case of serum TIMP-1, the best cut-off value was 102.5 µg/L with 43% sensitivity and 95% specificity, producing AUC = 0.77 with AUC 95% confidence interval of 0.6725 to 0.8780 and *p=0.0001*. Concerning the serum survivin/TIMP-1 ratio, the best cut-off value was 19.1 with 66% sensitivity and 95% specificity, producing AUC = 0.86 with AUC 95% confidence interval of 0.7931 to 0.9279 and *p<0.0001.*

3.4 Immunoblotting Detection of Survivin and TIMP-1 Proteins

Western blot results showed over-expressions in both survivin (Fig. 2A) and TIMP-1 (Fig. 2B) proteins with the progression of hepatic fibrosis.

3.5 RT-PCR Assessments of Survivin and TIMP-1 mRNA Levels

RT-PCR results revealed a gradual increase in the mRNA levels of both survivin (Fig. 2C) and TIMP-1 (Fig. 2D) with the progression of liver fibrosis.

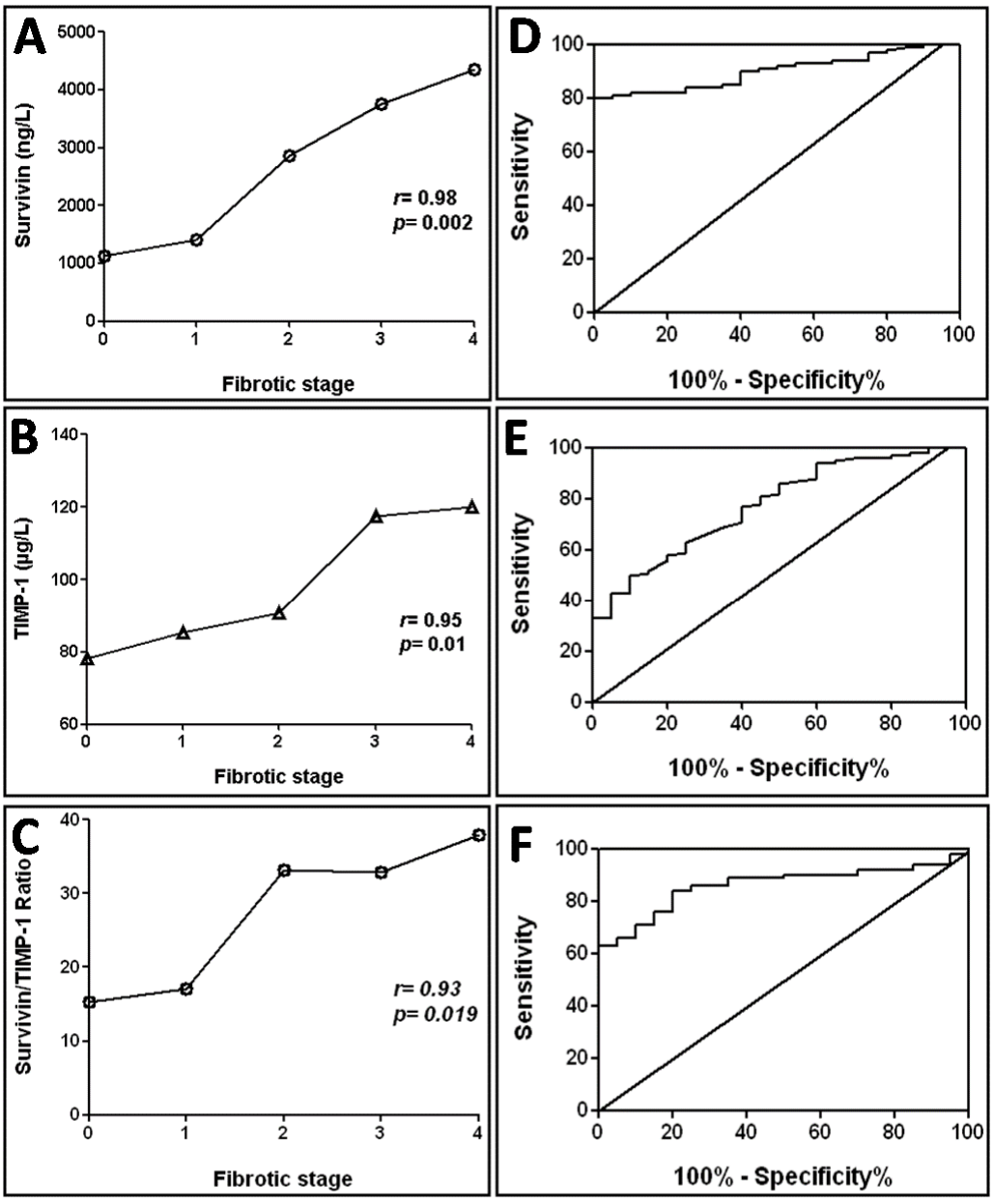

Fig. 1. Correlations between the fibrotic stage and each of serum survivin (A), TIMP-1 (B), and survivin/TIMP-1 ratio (C) in the studied patients. D, E, and F are ROC curves for serum survivin, TIMP-1, and survivin/TIMP-1 ratio, respectively in liver fibrosis

Table 2. Clinical, demographic and biochemical data of patients and controls

	Control	Group-I	Group-II	Group-III	Group-IV	Group-V
Fibrotic stage	Healthy	F0	F1	F2	F3	F4
Sex (M/F)	14/6	13/7	11/9	15/5	12/8	10/10
Age (year)	44.8±7.4	47.4±7.0	50.4±7.1	52.5±8.3*	53.8±7.7**	57.7±6.3***,†††,‡
Viral load (IU/ml)×10^3	Undetectable	208±175	202±156	468±344	6792±3754†††,‡‡‡,§§§	2236±2058$^{\dagger,\ddagger,\S,\bullet\bullet\bullet}$
sALT (U/L)	12.6±2.9	16.4±2.9	39.1±7.1***,†††	43.2±12.5***,†††	44.8±13.4***,†††	49.4± 15.9***,†††,‡,
sAST (U/L)	13.9±3.8	36.4±7.3**	42.1±16.5***	64.7±21.3***,†††,‡‡	81.0±38.7***,†††,‡‡‡	78.6±12.3***,†††,‡‡‡
Total bilirubin (µmol/L)	11.3±2.9	13.5±3.4	13.7±3.9	14.7±3.2	15.0±3.6*	19.5±5.5$^{***,\dagger\dagger\dagger,\ddagger\ddagger\ddagger,\S\S,\bullet\bullet}$
Serum albumin (g/L)	47.7±4.0	39.9±3.4**	40.2±1.7**	38.7±2.5***	40.0±5.7***	40.6±6.2***
Serum survivin (ng/L)	851±200	1135±329	1403±567	2870±775***,†††,‡‡‡,	3764±1291$^{***,\dagger\dagger\dagger,\ddagger\ddagger\ddagger,\$}$	4350±1373$^{***,\dagger\dagger\dagger,\ddagger\ddagger\ddagger,\$\$\$}$
Serum TIMP-1 (µg/L)	74.1±18.6	78.2±14.9	85.4±13.6	90.7±20.6	117.4±13.3$^{***,\dagger\dagger\dagger,\ddagger\ddagger\ddagger,\$\$\$}$	120.1±25.1$^{***,\dagger\dagger\dagger,\ddagger\ddagger\ddagger,\$\$\$}$
Survivin/TIMP-1 Ratio	12.0±3.6	15.3±6.0	17.1±8.0	33.1±11.2***,†††,‡‡‡	32.9±13.4***,†††,‡‡‡	38.0±14.1***,†††,‡‡‡

*Data are presented as mean ± SD (n = 20). *, †, ‡, § and ● indicate significant changes from control, F0, F1, F2 and F3 groups respectively. *, †, ‡, § and ● indicate significant change at p<0.05; **, ††, ‡‡, §§ and ●● indicate significant change at p<0.01; ***, †††, ‡‡‡, §§§ and ●●● indicate significant change at p<0.001. sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; TIMP-1, tissue inhibitor of the metalloproteinase-1.*

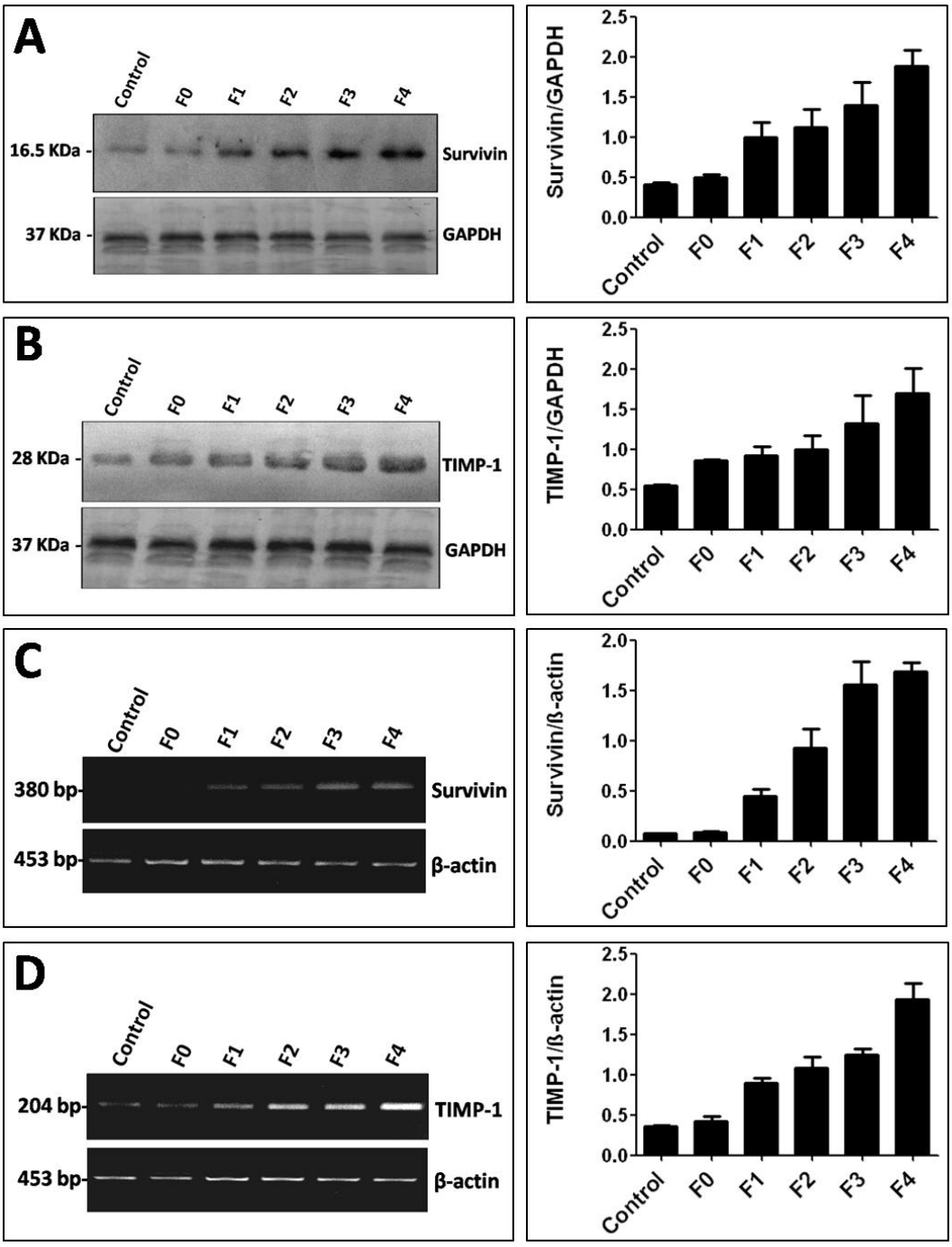

Fig. 2. Illustration of the Western blot assessments of survivin (A) and TIMP-1 (B) as well as RT-PCR detection of mRNA fragments of survivin (C) and TIMP-1 (D) in different stages of liver fibrosis. GAPDH and β-actin were used in parallel as internal controls. The right panels represent the corresponding quantification of each analysis measured by Image J software and expressed as the relative band density to the GAPDH or β-actin (Data are presented as mean ± SD (n = 3). GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; TIMP-1, Tissue inhibitor of the metalloproteinase-1

Table 3. Pearson's correlations between different parameters and each of serum surviving (ng/L) or TIMP-1 (µg/L)

	Survivin		TIMP-1	
	***r* value**	***p* value**	***r* value**	***p* value**
sALT	0.64	***	0.48	***
sAST	0.71	***	0.46	***
Total bilirubin	0.44	***	0.33	***
Serum albumin	-0.25	**	-0.15	NS
Viral load	0.49	***	0.45	***
Fibrotic stage	0.98	**	0.95	*
Survivin	-	-	0.51	***

** indicates significant correlation at p<0.05; ** indicate significant correlation at p<0.01 and *** indicate significant correlation at p<0.001. NS indicates non significant correlation. sALT, serum alanine aminotransferase; sAST, serum aspartate aminotransferase; TIMP-1, tissue inhibitor of the metalloproteinase-1.*

4. DISCUSSION

Liver fibrosis, the scarring of the hepatic parenchymal tissues, is the common final pathway of almost every chronic inflammatory liver injury which may accumulate and ultimately lead to the formation of cirrhosis with serious complications such as portal hypertension, hepatic synthetic impairment and HCC [22,23]. For patients with chronic liver diseases, including HCV, determination of the severity of hepatic fibrosis is considered as a routine procedure to dictate the need and timing of therapy, and may also define treatment response [22]. Since decades, this assessment of hepatic fibrosis has traditionally been achieved by histopathological examinations of a liver biopsy. However, the invasiveness of the liver biopsy procedures is considered as a serious drawback that increases the risk of potential complications including bleeding which ranges from 0.3% to 0.5%, and mortality for up to 0.1% [24,25]. Additionally, since the biopsy core only represents 1/20000 to 1/50000 of the whole liver tissues, biopsy is prone to a sampling error and variability that could lead to unfaithful appreciations of the fibrosis staging [11,26,27]. For these reasons, alternative non-invasive methods for accurate evaluation of liver fibrogenesis are of the pressing medical needs.

Some research groups investigated the usefulness of serum activities of sALT and sAST as reproducible fibrotic biomarkers. Both sALT and sAST showed obvious variations in different stages of liver fibrosis and therefore they were considered as useful indicators of liver fibrosis [28-30]. These results are in harmony with our findings where sAST and sALT were correlated with the fibrosis score although the sALT correlation was not statistically significant which could be attributed to the small sample size of the present study.

Other researchers investigated the variability of serum albumin and total bilirubin in different stages of liver fibrosis and concluded that neither of them could be considered as a dependable marker for monitoring liver fibrosis [31]. These findings are also confirmed in our work where the recorded changes in both serum albumin and total bilirubin levels were not significantly correlated with the fibrotic stage. Additionally, no significant correlation was found in our results between the viral load and the fibrotic stages.

Survivin is a 16.5-kDa protein that inhibits apoptosis and regulates cell division and proliferation. The expression of survivin is found to be undetectable or at very low levels in normal tissues, whereas it is found at high levels in embryonic and fetal tissues, in transformed cells, during HSCs activation and in various malignancies, including liver cancer [19,32-34].

In the current study, circulating levels of survivin showed a stepwise increase along the progression of hepatic fibrosis with a significant positive correlation with the fibrotic stage. Also, significant correlations were found between serum survivin and the other laboratory indices including sALT, sAST, total bilirubin, serum albumin, and the viral load as well as the patient's age.

Moreover, survivin protein and gene expressions confirmed the above described results and stepwise up-regulations of the survivin protein and mRNA fragment were recorded with the advancement of hepatic fibrosis from F1 to F4 compared to the controls. The results of the current study are in accordance with many

studies in which survivin up-regulation has been described in liver diseases that are accompanied by HSCs activation, suggesting a potential role for survivin in protecting HSCs from apoptosis during hepatic fibrogenesis [19,33]. However, survivin exhibits a role in regulating the survival of the normal adult cells [35].

Similarly, the current study also evaluated TIMP-1 as a biochemical serum marker of liver fibrosis. TIMP-1 is a 28 kDa protein that can irreversibly bind to and inactivate MMPs which are associated with matrix degradation [15]. It was reported that TIMP-1 is induced during liver injury [36]; Where it plays an important role in promoting liver fibrosis [17,37] and inhibiting liver regeneration [16].

In the present study, circulating levels of TIMP-1 were significantly increased in the advanced stage of liver fibrosis in comparison to the healthy control group, whereas its increased levels in the early stages of liver fibrosis did not reach the statistical significance. However, TIMP-1 levels in those patients showed a positive correlation with the fibrotic stage. In respect to the other laboratory indices, serum TIMP-1 levels showed significant correlations with sALT, sAST, total bilirubin, and the viral load as well as the patient's age.

Additionally, the results of the current study revealed up-regulation of TIMP-1 protein and mRNA fragment with the promotion of liver fibrosis stage where they are moderately up-regulated during mild and moderate fibrotic stages followed by a marked up-regulation during the advanced stage. These observations are in agreement with the results of previous studies which reported TIMP-1 correlations with the histopathological fibrotic scores [38,39]. These results clearly reflect the profibrogenic effects of TIMP-1 which may be mediated via preventing collagen degradation. This in-turn occurs through the inhibition of MMPs and protecting against activated HSCs death [40,41]. Rationally, this could elucidate the cooperative role of survivin and TIMP-1 in the fibrotic process and explain the significant positive correlation between serum survivin and serum TIMP-1 which has been recorded in the current study.

In contrast to the subtle increase in the early stages of liver fibrosis, the observed dramatic up-regulation of TIMP-1 during the advanced stages of liver fibrosis as shown in the present study can be attributed to the increased MMPs expressions by HSCs at the early stage of liver fibrosis, causing the liver ECM to degrade, while at the advanced stages, the fully activated HSCs over-express TIMPs especially TIMP-1 that inhibits MMPs activities [17,42,43]. This increased production of TIMPs relative to MMPs may be the important factor in the progression of liver fibrosis [44]. On the other hand, the values of survivin/TIMP-1 ratio were significantly higher in the moderate and advanced fibrotic stages than in the healthy controls, non fibrotic and mild fibrotic stages.

When assessing the diagnostic performance of circulating survivin, TIMP-1 and survivin/TIMP-1 ratio in discriminating liver fibrosis patients, survivin and survivin/TIMP-1 ratio showed better sensitivity over TIMP-1 with a superiority of survivin over survivin/TIMP-1 ratio. This indicates the predictive role of survivin in discriminating liver fibrosis stages and suggests its use as a potential non invasive marker for the early liver fibrosis. While mild liver fibrosis is largely asymptomatic, there is urgent need for measures that may induce regression of these fibrotic changes before its progression into cirrhosis and organ failure. These liver fibrotic changes in HCV patients are recently proved by some investigators to be reversible [45-47], if the underlying liver injury is successfully treated [48]. Thus, survivin could also act as a target for antifibrotic drugs for liver fibrosis and/or cirrhosis.

5. CONCLUSION

The findings of the present study indicated that survivin and TIMP-1 could be used as promising biomarkers for prediction and discrimination of HCV-associated liver fibrosis patients, with a priority for survivin. Circulating survivin could be considered as a potential valuable tool for the detection of the moderate/advanced stages (F2-F4) of liver fibrosis in HCV-infected patients with high sensitivity and specificity. Moreover, survivin could be used as a target for the antifibrotic drugs that may induce regression of liver fibrosis and/or cirrhosis that is recently proved to be reversible processes. In addition, survivin/TIMP-1 ratio can be used as a diagnostic marker for differentiating the early and advanced stages of liver fibrosis in HCV-infected patients. Further studies with validated tests on a large scale prospective clinical trial are required to ascertain these results.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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