

Expression of Beclin-1, Bcl-2, Bcl-xL, Bad, and Bax in HCV Patients in Relation to Grade of Hepatic Fibrosis

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Abstract

Autophagy plays an important role in the pathogenesis of many diseases. However, its role is still unclear. We investigate the mRNA expression of Beclin-1 (major autophagic agent), pro-apoptotic agents (Bad, Bax), and anti-apoptotic agents (Bcl-2, Bcl-xL) in blood samples withdrawn from Genotype 4 HCV-infected patients with different stages of hepatic fibrosis. The study was a retrospective one that included 30 healthy people (**Control Group**), 64 chronic hepatitis C patients with early hepatic fibrosis stages [grade 0 and 1 fibrosis] (**F0-1 Group**), and 36 chronic hepatitis C patients with Late hepatic fibrosis stages [grade 2 and 3 fibrosis] (**F2-3 Group**). qPCR was used to measure mRNA expression in the samples. Beclin-1, Bad, and Bax mRNA expression in F0-1 Group were significantly higher than both F2-3 Group and Control Group ($P < 0.001$). While Bcl-2, and Bcl-xL mRNA expression in F0-1 Group were significantly lower than both F2-3 Group and Control Group ($P < 0.001$). Beclin-1, Bad, and Bax mRNA expression were increased at the early stages of hepatic fibrosis in HCV patients, and were declined as the fibrosis progressed to more advanced stages, while Bcl-2, and Bcl-xL mRNA expression were increased as fibrosis progresses. This shows that Autophagy has an important role in the early stages of hepatic fibrosis in Genotype 4 HCV patients. These findings provide an insight into the pathogenesis of chronic HCV infection, and the effect of autophagy on liver fibrosis. This may be used to provide possible biomarkers and contribute to a new therapeutic approach.

Keywords: Autophagy, Liver fibrosis, Hepatitis C virus, Beclin-1, Bcl-2, Bcl-xL, Bad, Bax

Autophagy is a metabolic process in which the cellular components are degraded. This process involves the sequestering of these components inside a double-membrane vesicle called the Autophagosome then the formed Autophagosomes are fused with lysosomes forming Autophagolysosomes in which degradation occurs. Autophagy is induced in periods of nutrient deprivation to maintain the cellular homeostasis. It may also lead to autophagic cell death which makes it double-edged sword. Autophagy Disruption has been found to be involved in many conditions such as ageing, cardiomyopathy, neurodegeneration, liver diseases, and carcinogenesis. Moreover, Autophagy has a strong relationship to Apoptosis. It has been found that Autophagy induction suppresses Apoptosis, and Apoptosis Induction suppresses Autophagy. In addition, many of the Apoptosis components have a role in Autophagy as well [1-4].

Hepatitis C Virus (HCV) is a global health burden. About 170 million

people are infected with HCV worldwide.[5] Egypt has a very high HCV prevalence. Genotype 4 is the predominant genotype of HCV in Egypt. It is estimated that 10% of the Egyptian population is positive for HCV RNA [6]. More than 50% of HCV infections often progress to liver steatosis, fibrosis, cirrhosis, and ultimately to hepatocellular carcinoma (HCC) [5].

HCV has been found to induce autophagy which promotes the viral growth via regulating its RNA replication, translation of viral RNA, and assembly of viral particles. Therefore, suppressing this autophagic process suppresses HCV replication, which makes it an important target for HCV therapy [5].

In this study, we investigate the mRNA expression of major autophagic components such as **Beclin-1**, Bcl-2-associated death promoter (**Bad**), Bcl-2-associated X protein (**Bax**), B-cell lymphoma 2 (**Bcl-2**), and B-cell lymphoma extra-large (**Bcl-xL**) in Genotype 4 HCV infected patients with different stages of hepatic fibrosis and their relationship to other Clinicopathological features and their

prognostic significance. We also investigate the correlations, if any, among the autophagic components under study.

Patients And Methods

Patients

This retrospective study was comprised of a control group and two groups of Egyptian patients infected with chronic hepatitis C. They were selected from the outpatient clinic of Kasr al Ainy, University hospital, Cairo University between the periods of May 2015 to December 2015. All patients who were 18 years old or above, were included in the study. Only patients with HCV genotype 4 were included in this study.

The first group (Control group) was comprised of 30 healthy patients (18 male, 12 female, mean age 36.4±8.3, and range 24-55)

The second group was comprised of 64 chronic hepatitis C patients with early fibrosis stages [grade 0 and 1 fibrosis] (F0-1 Group). All of them were HCV positive (42 male, 22 female, mean age 40.1±9.2, and range 18-57).

The third group was comprised of 36 chronic hepatitis C patients with significant fibrosis stages [grade 2 and 3 fibrosis] (F2-3 Group). All of them were HCV positive (23 male, 13 female, mean age 42.6±10.8, and range 22-60).

Diagnosis of HCV patients was on basis of HCV-RNA positivity using polymerase chain reaction (PCR).

The study protocol conformed to the ethical guidelines of Research Ethics Committee for experimental and clinical studies at Faculty of Pharmacy - Cairo University, and was approved by the committee before the start of the research.

Patients and control subjects were asked to give written informed voluntary consent to participate in the study, and to accept the publication of their results.

Sampling Method and Storage

Peripheral venous blood samples were obtained from the patients and control group, after obtaining their informed consent along with their clinical history. They were all subjected to physical examination and clinical investigations, mentioned below. Then, samples were immediately stored at -80°C.

Virological and Chemical Assessment

HCV-RNA viral load was determined using Real-Time PCR. Also, Fibrosis stage and Steatosis level were determined according to Kleiner et al. HCV Genotype was determined by real-time PCR [7].

Stage of fibrosis was assessed using Fibroscan. Liver stiffness measurements (LSM) were expressed in kilopascals (kPa). Interquartile range (IQR) was defined as an index of intrinsic variability of LSM values corresponding to the interval of the LSM results containing 50% of the valid measurements between the 25th and 75th percentiles. As suggested by the manufacturer ten successful acquisitions were performed on each patient. The median value was considered representative of the elastic modulus of the liver. Only procedures with at least 10 valid measurements,

a success rate of at least 60%, and an interquartile range (IQR/M) <30% were considered reliable. FibroScan failure is defined when less than 10 valid measurements are obtained.

Other Investigations included: Complete Blood Count (CBC), serum creatinine, Alanine aminotransferase (ALT), and Aspartate aminotransferase (AST), Alkaline Phosphatase (ALP), Serum Albumin, total Bilirubin, prothrombin time, prothrombin concentration, and HCV Antibody.

Total Nucleic acid extraction

Total Nucleic acid extraction was done for the whole blood samples by MagNA Pure LC Instrument using MagNA Pure LC Total Nucleic acid isolation Kit Cat. No. 03038505001 (Roche Diagnostics GmbH, Roche Applied Science Mannheim, Germany). For the isolation, 200 µl of the whole blood sample was used to produce final elution volume of 50 µl. Reagents were added to the MagNA Pure LC Instrument as indicated by the manufacturer.

Reverse Transcription method and storage

cDNA synthesis from the isolated RNA was performed using Transcriptor First Strand cDNA Synthesis Kit Cat.No.04896866001 by (Roche Diagnostics GmbH, Roche Applied Science Mannheim, Germany). Reverse transcription was done using 11 µl of the extracted Total nucleic acid in the presence of 2 µl of the random hexamer primer, 4 µl of the Transcriptor reverse transcriptase reaction buffer, 0.5 µl of the RNase inhibitor, 1mM of each of the dNTPs (dATP, dTTP, dCTP, dGTP), and 0.5 µl of the reverse transcriptase.

The reverse transcription was performed at 25°C for 10 min, 55°C for 30 min, and 85°C for 5 minutes in a Roch LightCycler 2.0 Instrument. The cDNA samples were stored in -20°C.

Primers and real-time PCR

Oligonucleotide primers were used according to Kotsafti et al [8]. Primers were synthesized by TIB MOLBIOL syntheselabor GmbH, Berlin, Germany. Nucleotide sequences for the forward and reverse primers used for real-time PCR are mentioned in Table A.

Table A. Nucleotide sequences for the forward and reverse primers used for Beclin-1, Bad, Bax, Bcl-2. Bcl-xL, β-actin in the real-time PCR

Target name	Forward Primer	Reverse Primer	Amplicon length
Beclin-1	5'-GAGGGATGGA AGGGTCTAAG-3'	5'-GCCTGGGCT GTGGTAAAGT-3'	159 bp
Bcl-2	5'-GGATCCAGGAT AACGGAGGC-3'	5'-CCAGATAGG CACCCAGGGT-3'	147 bp
Bcl-xL	5'-GGCAGGCGACG AGTTTGA-3'	5'-CCCATCCCGG AAGAGTTCAT-3'	127 bp
Bad	5'-GGAGGATGAGT GACGAGTTGTG-3'	5'-GGGTGGAGTT TCGGGATGT-3'	193 bp
Bax	5'-CTTTTGCTTCAG GGTTTCATCC-3'	5'-TTGAGACACT CGCTCAGCTTCT-3'	119 bp
β-actin (House Keeping Gene)	5'-CCTGGCACCCA GCACAA-3'	5'-GCCGATCCAC ACGGAGTACT-3'	70 bp

Real-time PCR was implemented in Roch LightCycler 2.0 Instrument using LightCycler FastStart DNA MasterPLUS SYBR Green I Kit (Cat. No. 03515885001) by (Roche Diagnostics GmbH, Roche Applied Science Mannheim, Germany) [9].

The reactions were done in capillary tubes. Each tube contained a final volume of 20 µl containing 4 µl of SYBR Green Master Mix, 9 µl PCR grade water, 2 µl of the PCR primer mix, and 5µl of cDNA template. Capillary tubes were centrifuged in LC Carousel centrifuge 2.0.

After a pre-incubation step at 95°C for 10 min, samples underwent 45 cycles of 10s at 95°C and then: 15s at 66°C for all of Beclin-1, Bad, Bax, Bcl-2, Bcl-xL and β - actin. Finally, the last step was performed at 72°C for 25s.

Relative Quantitative analysis is automatically done by the LightCycler 2.0 software. Data were expressed a concentration ratio of the mRNA expression of the target gene in comparison to that of β-actin which was used as a housekeeping gene.

Statistical Analysis

Qualitative data were presented as numbers and percentages. Quantitative data were expressed as medians and ranges. Statistical analysis was performed using the SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed with the Kruskal-Wallis test. Two-tailed P value<0.05 was deemed to be significant. The relationship between two variables was determined using Spearman's rank correlation analysis. Determination of the mRNA expression cut-off value predicting late significant fibrosis stages was done using the receiver operating characteristics (ROC) curve.

Results

Beclin-1 mRNA expression

The Kruskal Wallis test indicated significant difference (P< 0.001) in Beclin-1 mRNA blood levels among the various groups except that there was no significant difference between Control and F2-3 Group (P=0.882) (Fig. A.1). F0-1 Group had the higher Beclin-1 mRNA levels 98.4 (64.9-960) than in Control Group 8.6 (0-35) or F2-3 Group 5.8 (0.01-32.9).

Bad mRNA expression

The Kruskal Wallis test indicated significant difference (P< 0.001) in Bad mRNA blood levels among the various groups (Fig. A.2). F0-1 Group had the higher Bad mRNA levels 105.5 (40.2-2310) than in Control Group 30.1 (0-80.1) or F2-3 Group 5.9 (0.7-64.1).

Bax mRNA expression

The Kruskal Wallis test indicated significant difference (P< 0.001) in Bax mRNA blood levels among the various groups (Fig. A.3). F0-1 Group had the higher Bax mRNA levels 99.1 (44.1-1260) than in Control Group 0.1 (0-20.1) or F2-3 Group 23.7 (2.2-58).

Bcl-2 mRNA expression

The Kruskal Wallis test indicated significant difference (P< 0.001) in Bcl-2 mRNA blood levels among the various groups (Fig. A.4). Control group showed the highest Bcl-2 mRNA levels 117 (9-712) compared to F0-1 Group 0.03 (0-8.1) or F2-3 Group 7.7 (0.04-198).

Bcl-xL mRNA expression

The Kruskal Wallis test indicated significant difference (P< 0.001) in Bcl-xL mRNA blood levels among the various groups (Fig. A.5). F2-3 Group had higher Bcl-xL mRNA levels 92.7 (10.3-1570) than in Control Group 11.5 (0-34) or F0-1 Group 0.2 (0-7.1).

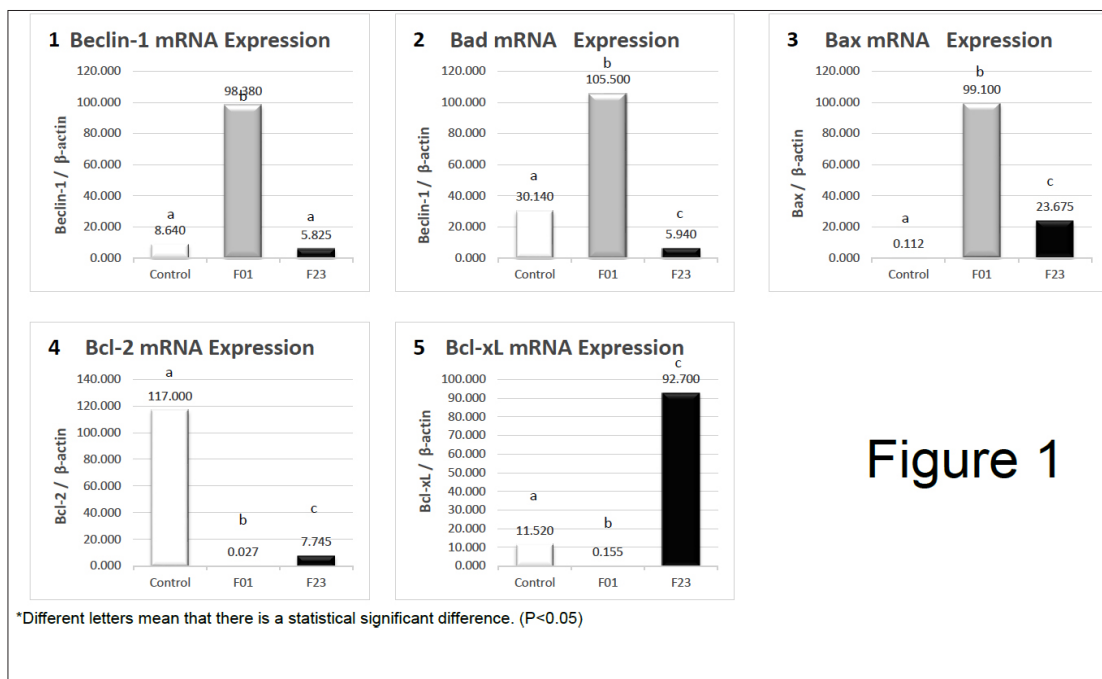


Figure 1

Figure A. Differences between groups in mRNA expression of Beclin-1 (1), Bad (2), Bax (3), Bcl-2 (4), Bcl-xL (5) were analyzed using the Kruskal-Wallis test. Data are expressed as median of the concentration ratio of the gene of interest to that of β-actin.

Correlation Analysis

A significant positive correlation emerged between Beclin-1 and Bad mRNA blood levels in both control group ($r=0.6$; $P=0.01$), and F2-3 group ($r=0.79$; $P=0.01$), and when all samples were considered together ($r=0.83$; $P=0.01$) (Table B).

An inverse correlation was observed between Beclin-1 and Bcl-2 mRNA blood levels only in F0-1 group ($r=-0.3$; $P=0.05$), and when all samples were considered together ($r=-0.74$; $P=0.01$).

A positive correlation was found between Beclin-1 and Bcl-xL mRNA blood levels in Control group ($r=0.48$; $P=0.01$), while an inverse correlation was found in F0-1 group ($r=-0.29$; $P=0.05$) and in F2-3 group ($r=-0.607$; $P=0.05$).

Positive correlation was identified between Bad and Bax mRNA blood levels in F0-1 group ($r=0.73$; $P=0.01$) and in F2-3 group ($r=0.654$; $P=0.01$).

Also, an inverse correlation was observed between Bad and Bcl-xL mRNA blood levels in F2-3 Group ($r=-0.535$; $P=0.01$). However, there was a positive correlation between Bad and Bcl-xL mRNA blood levels in Control Group ($r=0.565$; $P=0.01$).

Moreover, a significant positive correlation was also observed between Bad and Bcl-2 mRNA blood levels in F2-3 group ($r=0.392$; $P=0.05$).

Table B. Spearman's rank correlation Analysis among Beclin-1, Bad, Bax, Bcl-2, and Bcl-xL in the studied groups (F0-1, F2-3, Control groups). Data used were the concentration ratio of the gene of interest to that of the house keeping gene.

Group			Beclin-1	Bad	Bax	Bcl-2	Bcl-xL
F0-1	Beclin-1	Correlation Coefficient					
		Sig. (2-tailed)					
	Bad	Correlation Coefficient	0.088				
		Sig. (2-tailed)	0.491				
	Bax	Correlation Coefficient	0.149	.725**			
		Sig. (2-tailed)	0.239	0.001			
	Bcl-2	Correlation Coefficient	-.305*	-0.057	-0.046		
		Sig. (2-tailed)	0.014	0.652	0.719		
F2-3	Bcl-xL	Correlation Coefficient	-.286*	-0.029	0.061	.726**	
		Sig. (2-tailed)	0.022	0.82	0.635	0.001	
	Beclin-1	Correlation Coefficient					
		Sig. (2-tailed)					
	Bad	Correlation Coefficient	.792**				
		Sig. (2-tailed)	0.001				
	Bax	Correlation Coefficient	.698**	.654**			
		Sig. (2-tailed)	0.001	0.001			
Control	Bcl-2	Correlation Coefficient	0.254	.392*	.491**		
		Sig. (2-tailed)	0.135	0.018	0.002		
	Bcl-xL	Correlation Coefficient	-.607**	-.535**	-.612**	-.494**	
		Sig. (2-tailed)	0.001	0.001	0.001	0.002	
	Beclin-1	Correlation Coefficient					
		Sig. (2-tailed)					
	Bad	Correlation Coefficient	.601**				
		Sig. (2-tailed)	0.001				
Control	Bax	Correlation Coefficient	0.159	-0.108			
		Sig. (2-tailed)	0.402	0.569			
	Bcl-2	Correlation Coefficient	-0.156	-0.221	-0.046		
		Sig. (2-tailed)	0.41	0.24	0.81		
	Bcl-xL	Correlation Coefficient	.477**	.565**	0.045	-0.264	
		Sig. (2-tailed)	0.008	0.001	0.812	0.159	

*. Correlation is significant at the 0.05 level (2-tailed).

** . Correlation is significant at the 0.01 level (2-tailed).

Determination of Cut-off Values

ROC Curve was used to determine the cut-off values of Beclin-1 and the other autophagic markers for predicting patients with significant fibrosis stages [stage 2 and 3] (Fig. B). It was found that the threshold values of 48.9 for Beclin1, 52.57 for Bad, 58.2 for Bax, 1.2 for Bcl-2, and 8.7 for Bcl-xL were the closest to the point with both maximum sensitivity and specificity, and were thereby selected as cut-off mRNA expression values. (Table C).

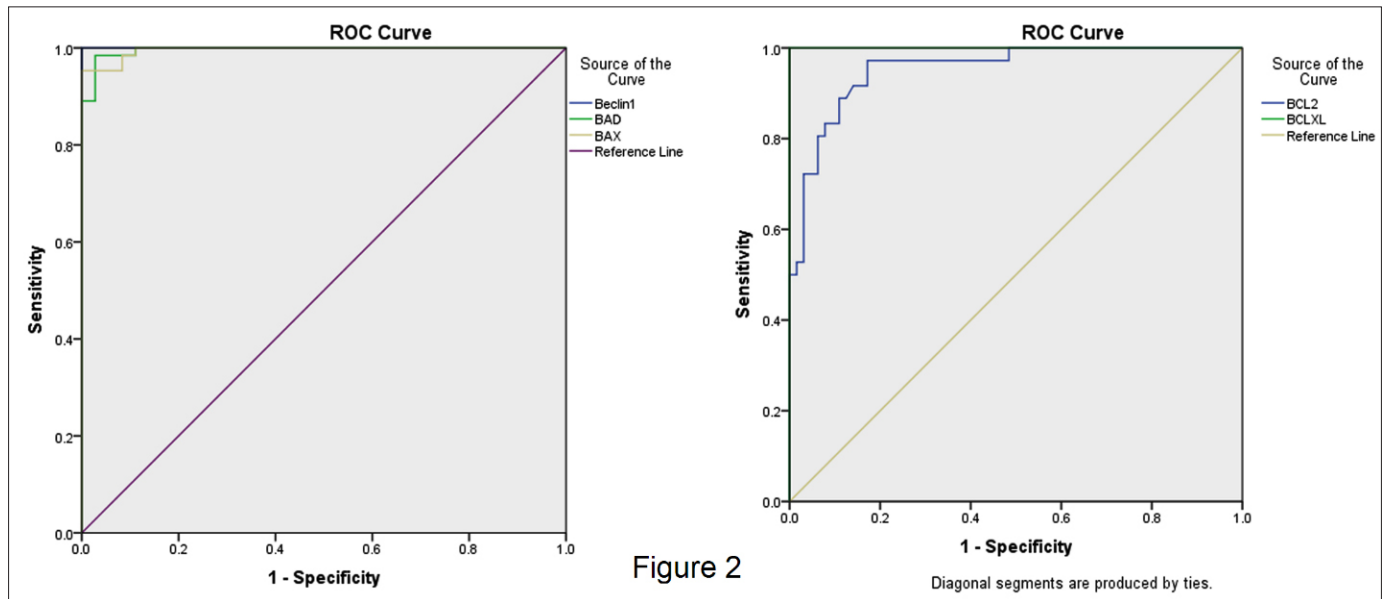


Figure 2

Figure B. ROC Curve used to determine the cut-off values of Beclin-1 and the other autophagic markers for late significant fibrosis [stage 2 and 3].

Table C. Cutoff values of Beclin1, Bad, Bax, Bcl2, and Bcl-xL for significant fibrosis [stage 2 and 3].

	Beclin-1	Bad	Bax	Bcl-2	Bcl-xL
Cut off value	48.9	52.57	58.2	1.2	8.7
Sensitivity	100%	98.4%	95.3%	97.20%	100%
Specificity	100%	97.2%	100.0%	82.80%	100%
95% Confidence Interval (CI)		(0.98-1)	(0.98-1)	(0.91-0.99)	
Area Under the Curve (AUC)	1	0.99	0.99	0.95	1

Discussion

In the presented study, we measured the mRNA expressions of the autophagic gene, beclin-1, pro-apoptotic components (Bad, Bax), and anti-apoptotic components (Bcl-2, Bcl-xL). Our aim was to study the effect of those biomarkers on the progression of hepatic fibrosis in Genotype 4 HCV-infected patients.

Beclin-1 is a main key protein involved in the induction of both autophagy and apoptosis [10]. Our findings showed that beclin-1 mRNA blood expression levels increase at the early stages of hepatic fibrosis in HCV patients, and declines as the hepatic fibrosis progresses to more advanced stages. Al-Shenawy et al [11]. studied the expression of beclin-1 mRNA in liver of HCV infected patients and found in contrast to our results that beclin-1 mRNA level increases as the fibrosis progresses to more advanced stages. There're two ways by which autophagy can promote or inhibit fibrosis. On one hand, autophagy can activate hepatic stellate cells which promotes hepatic fibrosis. On the other hand, autophagy can attenuate fibrosis by decreasing inflammation and supporting the

cells during stressful conditions [12]. Autophagy was shown to promote the HCV replication via regulation of RNA replication and assembly of the viral particles [4,5]. However, Autophagy was shown to be important only at the early phase of HCV infection, and it does not affect the late stages of HCV infected patients [13]. That was illustrated in our findings in which beclin-1 mRNA expression was at its highest level in F0-1 Group. Therefore, inhibiting Beclin-1 expression, and consequently autophagy, may be an important step in the search for a new therapeutic approach for hindering the progression of HCV infection at an early stage via blocking the viral replication.

Anti-apoptotic proteins such as Bcl-2, and Bcl-xL inhibit autophagy by binding to Beclin-1 preventing its action as an autophagy inducer [2,4,14]. In addition to that, they have role in inhibiting apoptosis via prevention of cytochrome C release from the mitochondria [8,15]. In the current study, we found that F0-1 group had the lowest Bcl-2 mRNA expression, and F2-3 group had higher level. In addition, it was shown that Bcl-xL level increases as the stage of fibrosis increases in the studied HCV patients (as previously reported in liver tissues by AL-Shenawy et al. [11]). All groups were expressing lower levels of Bcl-2 mRNA than the control group (in contrast to Hanafy et al. [16]). Piekarska et al [15]. found that, there was no correlation between Bcl-xL mRNA expression and degree of fibrosis in HCV patients, however, he found that the highest levels of Bcl-xL mRNA expression were in the patients with advanced stages of fibrosis, which is consistent with our findings.

Pro-apoptotic proteins such as Bad, and Bax were shown to induce autophagy in human cells via interacting with Bcl-2 which prevents its inhibitory effect on Beclin-1 protein [8,14]. Our results showed

that both Bad and Bax mRNA expression levels were inversely correlated with the stage of fibrosis with Group F2-3 showing lower mRNA expression levels than Group F0-1. Piekarska et al. [15] found similar results in liver biopsy specimens taken from chronic HCV patients. This supports, as previously mentioned, that autophagy activity is increased at the beginning of the disease, and it declines as the disease progresses towards more advanced stages. Therefore, it's anticipated that the use of autophagic inhibitors may be used in the treatment of HCV and HCV-associated liver fibrosis.

Our Observations showed that there was a positive correlation between Beclin-1 and Bad mRNA expression levels in all groups. That confirms that Beclin-1 and Bad work together for the induction of autophagy [14]. We found also that Beclin-1 mRNA expression levels were inversely correlated with Bcl-2 mRNA expression levels in all groups (In contrast to what was found by Al-Shenawy et al. [11]). This supports the role of Bcl-2 as an inhibitor of Autophagy [2]. These correlations can be of good use in understanding the pathogenesis, and developing new therapeutic approaches in cases of genotype 4, HCV infected patients.

Our analysis using ROC curves provided us with cut-off values for Beclin-1 and other autophagic markers, as mentioned before. Therefore, It can be predicted that HCV patients are more likely to have late-stage fibrosis if their mRNA expression levels were as follows; Beclin-1 < 48.9, Bad < 52.57, Bax < 58.2, Bcl-2 > 1.2, and Bcl-xL > 8.7.

Conclusion

Autophagy is a very important and rapidly growing aspect in HCV research. Nevertheless, it is still not completely clear regarding the functional mechanism of how autophagy affects the replication process of HCV, and how it affects fibrosis either positively or negatively. More studies are needed to further understand the mechanisms of these interactions and impacts of autophagy on the life cycle of HCV, as well as on liver fibrosis. These results shed the light on some key molecules controlling the pathway of HCV induced fibrosis, in an attempt to present possible biomarkers and new targets for HCV therapy and a better understanding of the interaction between Autophagy and liver fibrosis in Genotype 4 HCV patients. Also, these Autophagy associated mRNAs can be used as a biomarker for Genotype 4 HCV induced fibrosis.

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