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Livin Gene and *Yes Associted Protein 1 (YAP1)* Expression Are Markers for Bad Prognosis in HCV Associated Hepatocellular Carcinoma Patients

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Abstract: *Livin* gene and *Yes Associated Protein 1 (YAP1)* play a pivotal role in organ size control and tumorigenesis. In the present study, we investigated the expression of *Livin* gene and YAP 1 in HCV associated hepatocellular carcinoma (HCC) compared to other HCV patients and controls The studied patients were divided into three groups 30 patients in each group in addition to 30 healthy subjects as a control group. Relative quantification of *Livin* gene and YAP-1 was assessed by quantitative Real Time RT-PCR (qPCR) in all studied patients and healthy controls. Other laboratory investigations were done including CBC, INR, platelet number as well as liver function tests and tumour markers. Results displayed significant overexpression of *Livin* gene and YAP-1 detected in HCC group followed by HCV untreated group then HCV treated group. The relative quantitation (RQ) of both genes showed positive correlation to the carcinoembryonic antigen (CEA) level and a significant relation was found between higher level of *Livin* and YAP1 genes and tumor size. The overall survival rate was low in those patients with high levels of *Livin* and YAP 1 genes so they were considered as indicators of a bad prognosis. Conclusion: There is overexpression of *Livin* gene and YAP11 in hepatocellular carcinoma patients. They can be used as indicators of bad prognosis of the disease pathway together with low survival rate.

Key words: *Livin* · YAP1 · HCC and HCV

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and is the leading cause of mortality in patients [1]. An estimated half million new cases are diagnosed each year world-wide with disease burden highest in developing countries (85% of all cases) [2].

It was reported that 40%-80% of HCC cases have a positive HCV infection [3] and its highest incidence in the world was found in Egypt [4].

The inhibitors of apoptosis proteins (IAPs) are a family of functionally and structurally related proteins, which are closely associated with tumor occurrence and development. The IAP family consists of 8 members, termed baculoviral IAP repeat containing (BIRC) 1-8 and BRIC7, also known as *Livin* [5].

The *Livin* gene spans 4.6 kb on chromosome 20 at band q13. It is composed of six introns and seven exons. *Livin* protects cells from various pro-apoptotic stimuli by inhibiting the activity of caspase -3, -7 and -9 and it plays an important role in tumorigenesis and chemoresistance [6].

Livin is rarely detected in normal adult tissues but highly expressed in cancerous tissues. It is thought that *Livin* protein expression may be an early event in the occurrence of HCC [7].

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Yes Associated Protein 1 (YAP1) is a major downstream target of the Hippo-signaling pathway [8]. Regulation of the Hippo-signaling pathway is known to be mediated by phosphorylation and subcellular localization of YAP1. Activation of the Hippo-signaling pathway induces phosphorylation of YAP1, which prevents the translocation to the nucleus. When the Hippo-signaling pathway is inactivated, dephosphorylated YAP1 is translocated to the nucleus where it interacts with transcription factors, eventually leading to the proliferation of cells to various organ systems [9].

The aims of this study were to evaluate the expression levels of *Livin* gene and YAP1 in HCV associated HCC patients and their association to other laboratory parameters as well as the correlation of their expression levels with the overall survival rate in the HCC patients.

MATERIAL AND METHODS

This study is a case-control study. It was done by cooperation of Biochemistry department, Faculty of Science, Menoufia University, Medical Biochemistry & Molecular Biology and Microbiology departments, Faculty of Medicine, Menoufia University between December 2017 and June 2018 and included 90 patients and 30 healthy controls.

After taking informed written consent from all subjects and approval of the Ethical Committee of Medical Research- Menoufia Faculty of Medicine, all patients was subjected to the following: Full history taking, General and clinical examination, Ultrasound and C.T, laboratory investigations included: Complete liver function tests, Hepatitis markers, tumour markers including Alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA), Detection of gene expression by real time PCR. Patients with other types of hepatitis were excluded from the study. Subjects were classified into four groups: Group I: patients with Hepatocellular carcinoma on the top of chronic Hepatitis C (30 patients). Group II: patients with chronic Hepatitis C untreated (30 patients). Group III: patients with HCV who received treatment (30 patients). Group IV: apparently healthy control subjects (30 subjects).

Blood Samples: After overnight fasting ten-millimeter of venous blood was obtained from each participant and divided into three parts. First part 2 ml was put in citrated tube for use in detection of prothrombin time and INR. The second part 2 ml was put in EDTA tubes for complete blood count and total RNA extraction to be used in determination of *Livin* gene and YAP-1 gene expression. The remaining part was put in plain tube and left to stand for 10 minutes then centrifuged for 10 minute. The supernatant serum was put into several aliquots and stored at -80 to until used for determination of liver function tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin and direct bilirubin and tumor markers including AFP and CEA measurement by enzyme linked immunosorbent assay (ELISA - DRG International Inc., USA.) and detection of HCV-RNA presence by real-time polymerase chain reaction using COBAS TaqMan HCV quantitative test, version 2.0 (Roche Molecular Systems, Inc., Branchburg, NJ, USA) with a linear range from 43 to 69,000,000 IU/ml according to manufacturer instructions [10].

RNA Extraction and Quantitative Real Time PCR Assay of *Livin* and YAP1 Genes: Total RNA was isolated from whole blood using (The Invitrogen PureLink RNA Mini Kit), according to the manufacturer's' protocol. RNA quantification was conducted by Gene Quant II (Pharmacia Biotech) at 260 nm. Total RNA was stored at -80°C until molecular investigation was performed. 1 µg of total RNA from each sample was used for cDNA generation in a final reaction volume of 20 µl with High Capacity cDNA Archive Kit (Applied Biosystems).

The cDNA Amplification by Real-time PCR: The cDNA was used in SYBR green based quantitative real-time PCR for quantification of YAP1 and *Livin* gene expression by (Sensi FAST TM SYBR Lo-ROX Kit, Bioline), using the designed primers (Midland,TX). As shown in table A PCR was conducted under the following conditions: 95°C for 10 minutes, then 40 cycles; denaturation at 94 C for 15 sec, annealing at 60 C for 30 sec and extension at 72 C for 30 sec. Data analysis with Applied Biosystems 7500 software version 2.0.1 was carried out. The relative quantification (RQ) of gene expression completed using comparative ÄÄCt method where the amount of the target *Livin* gene and YAP 1 gene, are normalized to an endogenous reference gene (GAPDH) and relative to a control. Each run was completed using melting curve analysis to confirm specificity of the amplification and absence of primer dimers [11].

Table A: Primers used for detection of YAP1 gene and *Livin* gene

Gene	Primer	Accession number
YAP1 gene	Forward TAGCCCTGCGTAGCCAGTTA	
	Reverse TCATGCTTAGTCCACTGTCTGT	NM_001130145.3
Livin gene	Forward TGAGGAGTTGCGTCTGG	
	Reverse GCACGGCACAAAGACGAT	NM_139317.3
GAPDH	Forward TGCACCACCAACTGCTTAGC	
	Reverse GGCATGGACTGTGGTCATGAG	NM_002046.7

Statistical Methods: Data collected was analysed using SPSS version 23 computer statistical software package. The results were expressed as mean ± SD. The *ANOVA* F test was used to determine significant difference between test and control subjects. *Kruskal Wallis test*, Pairwise comparison between each 2 groups was done using Post Hoc Test (Dunn's test) for multiple comparisons test). Spearman coefficient was done for correlation between different studied parameters in each subject group. Cox regression of overall survival in HCC group was done for determination of hazard ratio**.** Statistical significance level was considered when $p<0.05$.

RESULTS

120 subjects were included in these study 90 patients and 30 healthy controls with 30 subjects in each of the studied group. No statistical significant difference was detected between different studied groups regarding demographic data or risk factors (Table 1). Figs. 1a & 1b show the amplification plot and melting curve of YAP1 and *Livin* genes expression respectively.

A high statistical significant difference was detected between the three studied groups and between all studied groups and control group regarding relative quantitation (RQ) of *Livin* and YAP1 genes expression levels with highest level was in HCC group followed by HCV untreated group then HCV treated group and control with median (8.76, 4.33, 0.78 and 0.78) of *Livin* gene and median of (9.42, 4.62, 4.70 and 0.55) in YAP 1 gene (Table 2 & Fig. 2).

Correlation between RQ of *Livin* gene expression and laboratory investigations in each group was estimated using Spearman coefficient method and the following were concluded from the results : there was a significant positive coefficient correlation between RQ of *Livin* gene expression with serum CEA and albumin levels in HCC group, while in HCV untreated group there was a significant coefficient negative association between RQ of *Livin* gene expression with serum creatinine level, BUN and INR level, also positive coefficient association was found between RQ of *Livin* gene expression and AFP serum level in HCV with treatment group ($p <0.05$) (Table 3).

The Correlation between RQ of YAP1 gene expression and laboratory investigations in each group was estimated with the following results : there was a significant positive coefficient correlation between RQ of YAP1 gene expression with serum CEA level in HCC group and negative association in HCV untreated group which demonstrated also a coefficient negative association between RQ of YAP1 gene expression with

Table 1: Comparison between the different studied groups according to demographic data and risk factors.

	HCC (n = 30)		HCV no treatment (n = 30)		HCV with treatment (n = 30)		Control (n = 30)			
	No.	%	No.	%	No.	%	No.	%	Test of Sig.	p
Gender										
Male	26	86.7	20	66.7	24	80.0	18	60.0	x^2=6.818	0.078
Female	4	13.3	10	33.3	6	20.0	12	40.0		
Age (years)										
Min.-Max.	35.0-65.0		19.0-70.0		38.0-68.0		30.0-68.0		F=2.459	0.066
Mean±SD.	51.03±7.64		49.77±16.34		56.87±7.83		49.47±14.0			
Median	53.0		55.0		56.0		45.0			
Risk factors										
Smoking	13	43.3	12	40.0	7	23.3	6	20.0	5.700	0.127
Diabetes	14	46.7	7	23.3	8	26.7	14	46.7	6.198	0.102
Hypertension	10	33.3	5	16.7	5	16.7	8	26.7	3.354	0.340

x^2: Chi square test, F: F for ANOVA test, p: p value for comparing between the studied groups

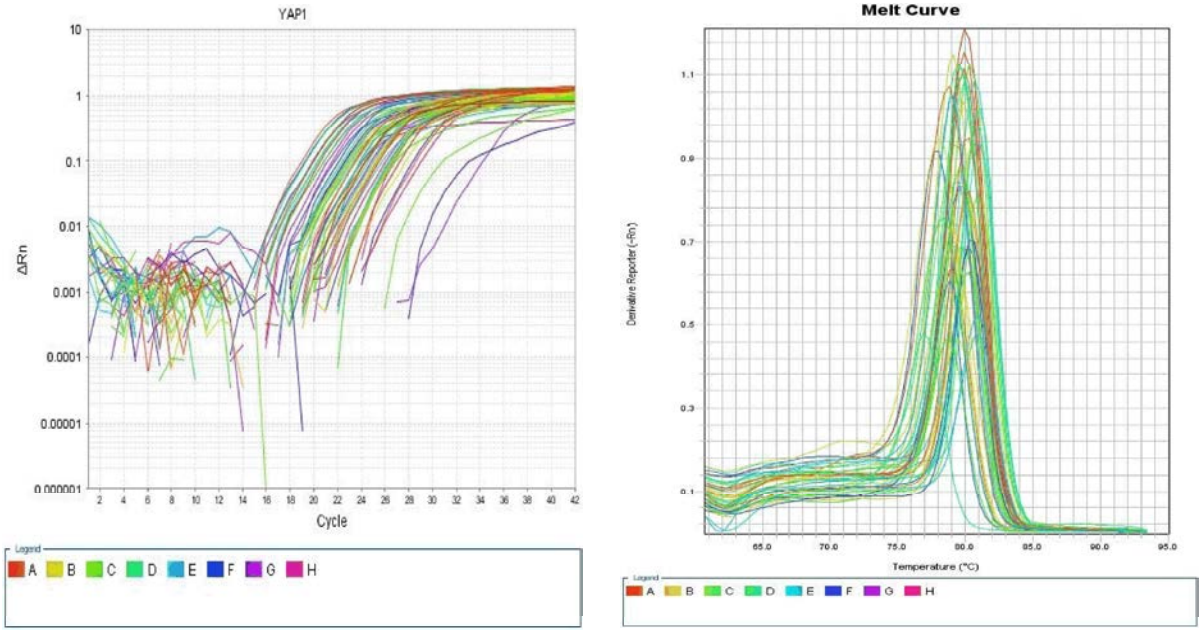

Fig. 1a: Amplification plot and melting curve of YAP1 gene expression

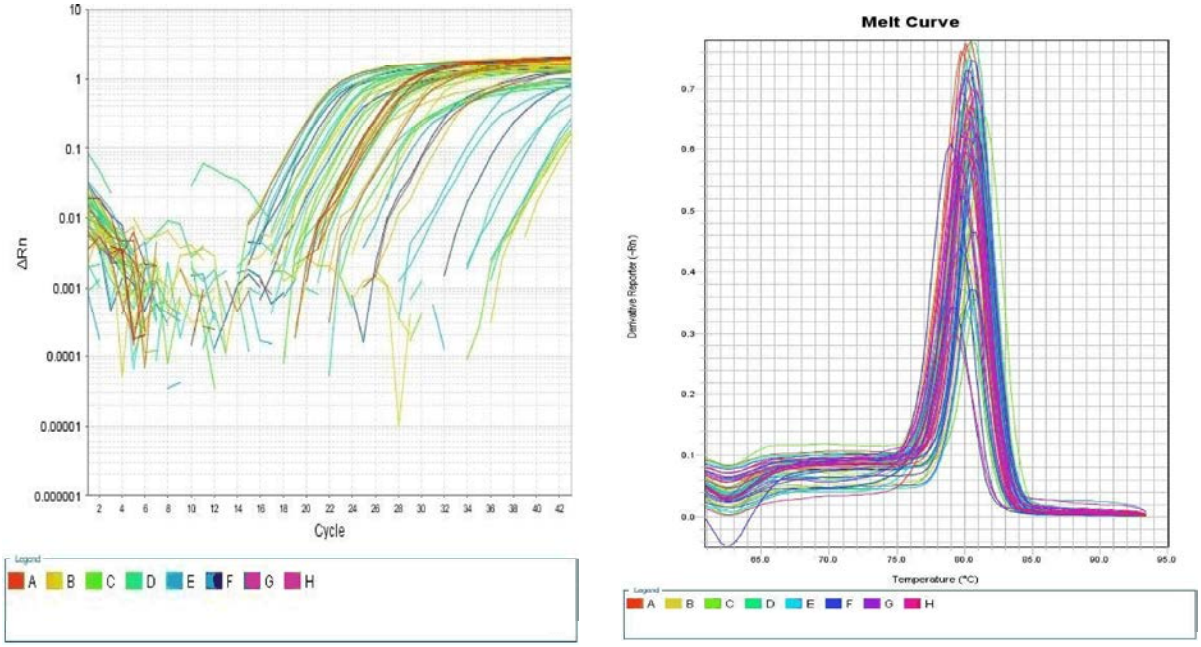

Fig. 1b: Amplification plot and melting curve of *Livin* gene expression

platelet count and positive association with prothrombin time. No association was found between RQ of YAP 1 gene expression with any of the studied parameters in HCV with treatment group and control group. (Table 4 & Fig. 3).

There was a significant difference between the RQ of *Livin* and YAP1 genes expression levels with different tumor size detected by US in HCC group with the highest levels in multifocal lesion, followed by tumor of diameter larger than 5 cm (Table 5).

The CEA level, RQ of *Livin* and YAP1 genes expression levels can be considered as a bad sign for overall survival in HCC patients by univariate analysis, while by multivariate analysis only RQ of YAP1 gene expression can be considered as bad sign for overall survival in HCC patients (Table 6).

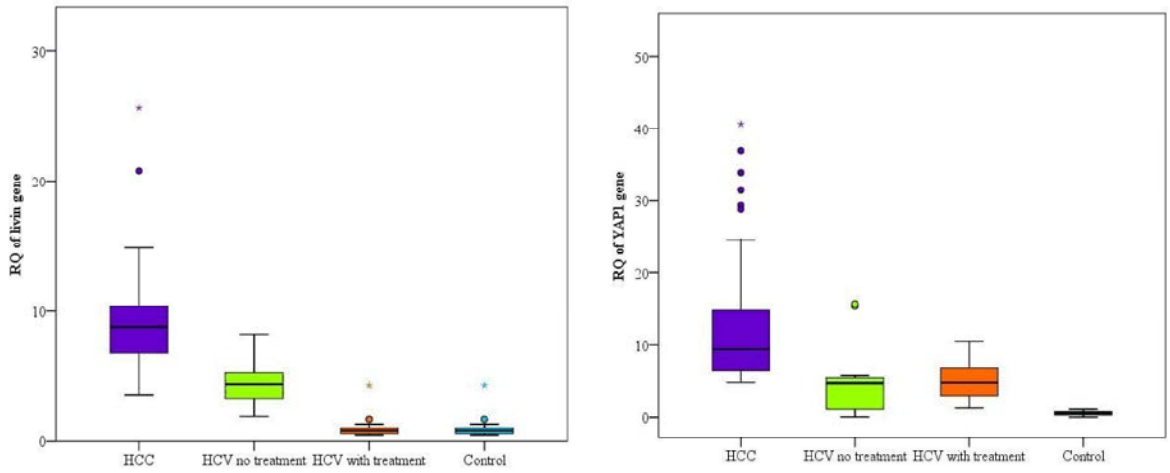

Fig. 2: Comparison between the different studied groups according to RQ of *Livin* and YAP1 genes

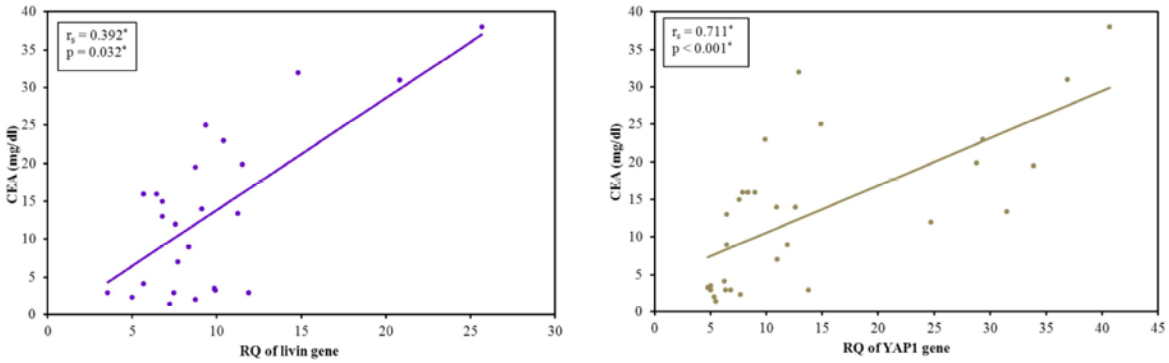

Fig. 3: Correlation between RQ of *Livin* and YAP1 genes and CEA (mg/dl) in HCC group

Table 2: Comparison between the different studied groups according to RQ

	HCC (n = 30)	HCV no ttt (n = 30)	HCV w ttt (n = 30)	Control (n = 30)	H	p
RQ of *Livin* gene						
Min.-Max.	3.54-25.67	1.89-8.20	0.43-4.25	0.43-4.25	95.201*	<0.001*
Mean±SD.	9.52±4.48	4.30±1.45	0.94±0.72	1.04±0.93		
Median	8.76	4.35	0.78	0.78		
p_1	<0.001*	<0.001*	0.873			
Sig. bet. Grps	p_2=0.002*,	p_3<0.001*,	p_4<0.001*			
RQ of YAP1 gene						
Min.-Max.	4.75-40.68	0.04-15.70	1.28-10.50	0.02-1.10	77.131	<0.001*
Mean±SD.	14.06±10.84	3.98±3.85	5.16±2.44	0.51±0.29		
Median	9.42	4.64	4.70	0.55		
p_1	<0.001*	<0.001*	<0.001*			
Sig. bet. Grps	p_2<0.001*,	p_3=0.001*,	p_4=0.112			

H: H for Kruskal Wallis test, Pairwise comparison bet. each 2 groups was done using Post Hoc Test (Dunn's for multiple comparisons test)

p: p value for comparing between the studied groups

p_1: p value for comparing between control and each other groups

p_2: p value for comparing between HCC group and HCV no ttt group

p_3: p value for comparing between HCC group and HCV w ttt group

p_4: p value for comparing between HCV no treatment group and HCV with treatment

*: Statistically significant at $p \leq 0.05$

Table 3: Correlation between RQ of *Livin* gene and laboratory investigations in each group

	RQ of *Livin* gene							
	HCC		HCV no ttt		HCV w ttt		Control	
	r_s	p	r_s	p	r_s	p	r_s	p
TLC (x10^3/ul)	-0.259	0.168	0.118	0.535	0.123	0.516	0.052	0.784
Platelets(x10^3/ul)	-0.003	0.986	-0.140	0.459	-0.019	0.921	-0.037	0.848
Prothrombin time percent	-0.146	0.443	0.148	0.435	0.126	0.508	0.069	0.718
INR	0.085	0.653	-0.482*	0.007*	-0.281	0.133	-0.026	0.891
ALT (IU/L)	0.217	0.248	-0.345	0.062	0.011	0.954	0.212	0.260
AST (IU/L)	0.059	0.757	-0.195	0.302	-0.215	0.253	0.073	0.701
ALP (IU/L)	0.138	0.468	-0.067	0.724	-0.131	0.491	-0.060	0.755
GGT (IU/L)	-0.366	0.046	-0.103	0.589	-0.090	0.636	-0.110	0.563
AFP (ng/ml)	0.358	0.052	0.163	0.391	0.449*	0.013*	-0.211	0.264
CEA (mg/dl)	0.392*	0.032*	-0.281	0.133	-0.357	0.053	0.093	0.625
Albumin (gm/dl)	0.451*	0.012*	0.287	0.124	-0.226	0.231	0.073	0.701
Total bilirubin (mg/dl)	-0.119	0.531	-0.115	0.544	0.063	0.740	0.184	0.330
Direct bilirubin (mg/dl)	0.170	0.370	-0.273	0.145	0.161	0.395	-0.095	0.618
BUN (mg/dl)	-0.053	0.779	-0.503*	0.005*	0.305	0.101	-0.011	0.955
Creatinine (mg/dl)	-0.320	0.084	-0.438*	0.016*	0.021	0.913	0.222	0.237

r_s: Spearman coefficient; *: Statistically significant at $p \leq 0.05$

Table 4: Correlation between RQ of YAP1 gene and laboratory investigations in each group

	RQ of YAP1 gene							
	HCC		HCV no ttt		HCV w ttt		Control	
	r_s	p	r_s	p	r_s	p	r_s	p
TLC (x10^3/ul)	-0.137	0.471	-0.024	0.898	0.135	0.478	-0.359	0.051
Platelets(x10^3/ul)	0.130	0.493	-0.528*	0.003*	0.302	0.105	-0.269	0.151
Prothrombin time percent	0.035	0.856	0.561*	0.001*	-0.184	0.331	-0.134	0.480
INR	0.012	0.949	-0.350	0.058	0.202	0.286	0.045	0.813
ALT (IU/L)	0.341	0.065	-0.069	0.716	-0.028	0.883	-0.004	0.984
AST (IU/L)	0.244	0.194	-0.085	0.656	0.030	0.877	-0.263	0.160
ALP (IU/L)	0.380	0.038	0.017	0.930	0.142	0.453	-0.348	0.060
GGT (IU/L)	-0.331	0.074	0.066	0.728	-0.248	0.186	0.190	0.314
AFP (ng/ml)	0.308	0.097	0.290	0.120	-0.182	0.335	-0.026	0.892
CEA (mg/dl)	0.711*	<0.001*	-0.606*	<0.001*	0.329	0.076	0.133	0.483
Albumin (gm/dl)	0.402	0.028	-0.120	0.529	-0.030	0.877	-0.263	0.160
Total bilirubin (mg/dl)	-0.171	0.367	0.199	0.291	0.195	0.302	-0.202	0.284
Direct bilirubin (mg/dl)	0.059	0.758	-0.031	0.870	0.100	0.598	0.206	0.275
BUN (mg/dl)	-0.087	0.646	0.030	0.874	-0.200	0.290	0.253	0.177
Creatinine (mg/dl)	-0.099	0.604	-0.035	0.856	-0.171	0.365	-0.026	0.892

r_s: Spearman coefficient; *: Statistically significant at $p \leq 0.05$

Table 5: Relation between RQ of *Livin* gene and RQ of YAP1 gene with tumor size by US in HCC group (n = 30)

		RQ of *Livin* gene				
Tumor size by US	N	Min.-Max.	Mean±SD.	Median	H	p
≤5	14	3.54-9.93	7.13±1.83	7.01	11.644*	0.003*
>5	9	5.67-20.80	11.37±4.45	11.53		
Multifocal	7	7.56-25.67	11.92±6.18	10.39		
		RQ of YAP1 gene				
Tumor size by US	N	Min.-Max.	Mean±SD.	Median	H	p
≤5	14	4.75-11.89	6.94±1.96	6.45	15.622*	<0.001*
>5	9	6.25-36.89	18.35±11.68	13.78		
Multifocal	7	9.89-40.68	22.79±11.94	24.67		

H: H for Kruskal Wallis test; p: p value for comparing between the different categories; *: Statistically significant at $p \leq 0.05$

Table 6: Cox regression of overall survival in HCC group for determination of hazard ratio

	Univariate		#Multivariate	
	p	HR (95%C.I)	p	HR (95%C.I)
Gender (female)	0.442	1.887(0.400-8.892)		
Age (years)	0.960	0.998(0.923-1.079)		
Smoking	0.712	0.788(0.222-2.796)		
Diabetes	0.616	0.724(0.204-2.564)		
Hypertension	0.463	1.607(0.453-5.701)		
Hb level (gm/dl)	0.383	1.158(0.833-1.608)		
TLC (x103/ul)	0.929	0.984(0.685-1.412)		
Platelets(x103/ul)	0.687	1.002(0.992-1.012)		
Prothrombin time percent	0.875	1.004(0.960-1.049)		
INR	0.951	0.908(0.044-18.963)		
ALT (IU/L)	0.610	1.007(0.980-1.035)		
AST (IU/L)	0.845	1.003(0.973-1.033)		
ALP (IU/L)	0.948	1.000(0.991-1.008)		
Albumin (gm/dl)	0.113	2.133(0.837-5.437)		
GGT (IU/L)	0.274	0.996(0.988-1.004)		
AFP (ng/ml)	0.883	1.000(0.999-1.001)		
CEA (mg/dl)	0.023*	1.074(1.010-1.142)	0.629	0.964(0.829-1.120)
Total bilirubin (mg/dl)	0.094	0.149(0.016-1.383)		
Direct bilirubin (mg/dl)	0.218	0.067(0.001-4.954)		
BUN (mg/dl)	0.969	1.002(0.928-1.081)		
Creatinine (mg/dl)	0.230	0.294(0.040-2.168)		
PCR $x10^5$	0.088	1.002(1.000-1.004)		
RQ of *Livin* gene	0.008*	1.168(1.402-1.308)	0.438	0.914(0.728-1.147)
RQ of YAP1 gene	<0.001*	1.124(1.065-1.186)	0.001*	1.196(1.078-1.328)

OR: Odd`s ratio, C.I: Confidence interval; #: All variables with p<0.05 was included in the multivariate; *: Statistically significant at p = 0.05

DISCUSSION

Despite great advances in diagnosis and treatment of HCC, the mortality rate is still high, especially in advanced stage. This indicates that a great effort is needed to identify novel prognostic markers and to develop new therapeutic strategies [12].

Livin is a member of the inhibitors of apoptosis proteins family, it plays a vital role in the regulation of apoptosis with subsequent modulation of cell cycle and cell proliferation. *Livin* is over-expressed in several cancer types, its anti-apoptotic activity is mediated mostly by the direct inhibition of caspase 3, 7 and 9 [13]. Our study proved that *Livin* gene was found to be significantly overexpressed in hepatocellular carcinoma patients, similar results were demonstrated by other authors [14]. *Livin* gene expression was also reported by many authors to be elevated in a number of other tumors like adrenocortical tumors, colorectal tumors [15, 16] superficial bladder cancer tumors [17], neuroblastoma [18] acute lymphoblastic leukemia [19] and melanoma as well as many other types of tumors [7].

Many studies concluded that IAP members as *Livin* gene represent attractive molecular targets for the design of new classes of anticancer drugs which can give promising results for treatment of many cancer patients [20, 21]. However the expression of this protein in many normal tissues may represent a challenge for its role in cancer therapy and represent a lot of side effects like nephrotoxicity, infertility and gastrointestinal disorders [22].

Yes Associated Protein 1 (YAP1) is a well-known oncogenic protein in human cancer [23]. It is a transcriptional regulator and it plays a pivotal role in organ size control [24]. In HCC, YAP1 was found to be overexpressed and could promote the growth and metastasis of HCC cells [25].

In our study significant overexpression of YAP-1 was detected in HCC patients as well as patients with HCV confirming the results which were reported by many other previous studies [26-28].

Correlation between RQ of both *Livin* gene and YAP1 expression and laboratory investigations in each group was estimated using Spearman coefficient method and

there was a significant positive coefficient correlation between RQ of *Livin* gene expression with serum CEA in HCC group also between RQ of YAP1 gene expression with serum CEA level in HCC group, this finding when added to the result that There was a significant difference between the RQ of *Livin* and YAP1 genes expression levels with different tumor size detected by US in HCC group with the highest levels in multifocal lesion, followed by tumor of diameter larger than 5 cm indicating that *Livin* and YAP1 genes are associated with HCC and indicating bad prognosis of the disease. Previous findings were reported by other studies like Fan *et al*. [29] who concluded that an increased expression of YAP-1 within PBMCs could serve as a bad indicator for the prognosis of HCC patients as that study reported high level of YAP1 in mononuclear cells and showed positive linear correlation to Treg percentage which is immunosuppressant cells [29].

The CEA level, RQ of *Livin* and YAP1 genes expression levels can be considered as bad signs for overall survival in HCC patients by univariate Cox regression analysis while by multivariate analysis only RQ of YAP1 gene expression can be considered as a bad sign for overall survival in HCC patients. This finding confirms what obtained by Zhang *et al*. [30] who demonstrated that high level of YAP 1 together with low level of miRNA-345 was was associated with low survival rate. Other study also reported that YAP-1 is associated with increased TGF-â within HCC and hyperplasia of oval cells together with activation of inflammatory cell infiltration and fibrosis [31].

Livin gene expression was also reported as a bad prognostic marker by Augello *et al*. [32] who concluded that *Livin* overexpression in HCC patients imply that its level could be used as a marker of cancer tissue and more importantly, could be related with patients' survival.

CONCLUSIONS

Based on the findings in our study, we concluded that there is overexpression of *Livin* and YAP 1 genes in hepatocellular carcinoma patients and HCV patients. So they can be used as indicators of bad prognosis of the disease pathway together with low survival rate in HCC patients. Future studies should focus on their patho -physiological role in progress of HCC as well as in other cancer types in order to develop new therapeutic choices.

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