A pilot study of Livin gene and Yes-associated protein 1 expression in hepatocellular carcinoma patients

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ABSTRACT

Background: Livin gene and Yes-Associated Protein 1 (YAP1) play a pivotal role in organ size control and tumorigenesis.

Aim: In the present pilot study, we investigate the expression of Livin gene and YAP1 in hepatitis C virus (HCV) associated hepatocellular carcinoma (HCC) compared to other HCV patients and controls.

Methods: 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 were assessed by quantitative Real Time RT-PCR (qPCR) in all studied patients and healthy controls. other laboratory investigations were done including complete blood count (CBC),international normalized ratio (INR) as well as liver function tests and tumor markers.

Results: Significant overexpression of Livin gene and YAP-1 was detected in HCC group followed by Hepatitis C Virus (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 YAP1 in hepatocellular carcinoma patients. They can be used as indicators of bad prognosis of the disease pathway together with low survival rate.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy and is the leading cause of mortality in patients (Alazawi et al., 2010). An estimated half million new cases are diagnosed each year world-wide with disease burden highest in developing countries (85% of all cases) (American Cancer Society, 2014).

It was reported that 40%–80% of HCC cases have a positive HCV infection (Mittal and El-Serag., 2013) and its highest incidence in the world was found in Egypt (Khattab et al., 2010).

A livin protein is a member of a large family of a related protein associated with tumor occurrence and development, called The inhibitors of apoptosis proteins (IAPs) which consists of 8 members, termed baculoviral IAP repeat containing (BIRC) 1–8. A BIRC7, also known as Livin (Vucic et al., 2000).

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 chemo resistance (Kasof et al., 2001).

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 (Lazar et al., 2012).

Yes-associated protein 1 (YAP1) is a major downstream target of the Hippo-signaling pathway (Lian et al., 2010). 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 (Liu et al., 2010).

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.

2. Subjects and methods

This pilot study is a case-control study. It was done by cooperation of Biochemistry department, Faculty of Science, Menoufa University, Medical Biochemistry & Molecular Biology and Microbiology departments, Faculty of Medicine, Menoufa 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- Menoufa Faculty of Medicine, all patients were subjected to the following: Full history taking, General and clinical examination, Ultrasound (US) and C.T, laboratory investigations included: Complete liver function tests, Hepatitis markers for Hepatitis A Virus (HAV): IgM for recent infection, IgG for old infection, for Hepatitis B Virus (HBV): HBsAg, HBsAb, HB core Ab, HBeAg, HBe Ab. for Hepatitis C Virus: HCV-Ab, PCR, kidney function tests, tumor 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 after making Hepatitis markers mentioned before from medical reports. Subjects were classified into four groups: Group I: patients with Hepatocellular carcinoma on the top of chronic Hepatitis C untreated (30 patients). Group II: patients with chronic Hepatitis C untreated after investigation include + HCV Ab,CT, tumor markers (30 patients). Group III: patients with HCV who received treatment (30 patients) in the form of Sofosbuvir 400 mg and Daclatasvir 60 mg daily for 12 weeks. During treatment, they were closely monitored at week 2, week 4, week 8, and week 12 by laboratory studies including CBC, creatinine, AST, ALT, and total bilirubin. Group IV: apparently healthy control subjects (a blood bank donors) (30 subjects).

Blood samples: after overnight fasting ten ml of venous blood was obtained from each participant and divided into three parts. First part two ml was put in citrated tube for use in detection of prothrombin time and international normalized ratio (INR). The second part two 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 min then centrifuged for 10 min at 4000 RPM. The supernatant serum was put into several aliquots and stored at -80 °C until used for determination of liver function tests 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) (Ghany et al., 2009).

2.1. 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 (SensiFAST 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 min, then 40 cycles; denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Data analysis with Applied Biosystems 7500 software version 2.0.1 was carried out. The relative quantification (RQ) of gene expression completed using comparative ΔΔCt 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 (Liu et al., 2013). Fig. 1a shows the amplification plot and melting curve of Livin gene expression. While Fig. 1b shows the amplification plot and melting curve of YAP1 gene expression.

| Table A | Primers used for detection of YAP1 gene and Livin gene. | Accession number |
|--------|--------------------------------------------------------|------------------|
| Livin gene | Forward TAGGAGATTGGTGGTCTGTT | NM_139317.3 |
| YAP1 gene | Forward TAGGGAGTGGTCTGTT | NM_001130145.3 |
| GAPDH | Reverse GCGAGGCAACAGGAGAT | NM_002046.7 |
| | Reverse GGCAATGGACTTGGTCATGAG | |

2.2. Statistical methods

Data collected was analyzed 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.

3. 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).

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 RQ of YAP1 gene expression, 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, Blood Urea Nitrogen (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 2).

The correlation of RQ of livin and YAP1 gene expression and viral load: there was a non significant correlation between viral load and both livin and yap1 gene expression in all groups (not shown).

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 platelet count and positive association with prothrombin time. No association was found between RQ of YAP1 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 ultrasound (US) in HCC group with the highest levels in multifocal lesion, followed by tumor of diameter larger than 5 cm (Table 5 & Fig. 4).

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

Fig. 1. (a): Amplification plot and melting curve of Livin gene expression. (b): Amplification plot and melting curve of YAP1 gene expression.
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).

4. 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 (Yin et al., 2008).
### Table 3
Correlation between RQ of Livin gene expression and laboratory investigation & RQ of YAP1 gene expression in each group.

| RQ of Livin gene expression | HCC   | HCV no ttt | HCV w ttt | Control |
|-----------------------------|-------|------------|-----------|---------|
|                             | rs    | p          | rs        | p       | rs      | p       |
| TLC (x10³/ul)               | -0.259| 0.168      | 0.118     | 0.535   | 0.123   | 0.516   | 0.052   | 0.784   |
| Platelets (x10³/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   |
| RQ of YAP1 gene expression  | 0.680 | 0.001      | 0.0246    | 0.897   | 0.1415  | 0.4557  | 0.196   | 0.299   |

r_s: Spearman coefficient.
* Statistically significant at p ≤ 0.05.

### Table 4
Correlation between RQ of YAP1 gene and laboratory investigation in each group.

| RQ of YAP1 gene | HCC   | HCV no ttt | HCV w ttt | Control |
|-----------------|-------|------------|-----------|---------|
|                 | rs    | p          | rs        | p       | rs      | p       |
| TLC (x10³/ul)   | -0.137| 0.471      | -0.024    | 0.698   | 0.135   | 0.478   | -0.359  | 0.051   |
| Platelets (x10³/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*     | -0.006*   | <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 ≤ 0.05.

![Fig. 3](image-url). Correlation between RQ of Livin and YAP1 genes and CEA (mg/dl) in HCC group.
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 (Altieri et al., 2017). Our study proved that Livin gene was found to be significantly overexpressed in hepatocellular carcinoma patients, similar results were demonstrated by other authors (Chio et al., 2015). Livin gene expression was also reported by many authors to be elevated in a number of other tumors like adenocortical tumors, colorectal tumors (Myung et al., 2013) & Wang et al., (2014) superficial bladder cancer tumors (Gazzaniga et al., 2003) neuroblastoma (Kim et al., 2005) acute lymphoblastic leukemia (Choi et al., 2013) and melanoma as well as many other types of tumors (Lazar et al., 2012).

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 (Wang et al., 2008) (Shiera et al., 2013). 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 (Ding et al., 2013).

YAP1 is a well-known oncogenic protein in human cancer (Harvey et al., 2013). It is a transcriptional regulator and it plays a pivotal role in organ size control (Zanconato et al., 2016). In HCC, YAP1 was found to be overexpressed and could promote the growth and metastasis of HCC cells (Farazi and DePinho, 2006).

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 (Xu et al., 2009) (Xu et al., 2013) (Zhang et al., 2012).

Correlation between RQ of both Livin gene and YAPI gene expression and laboratory investigations in each group was estimated using Spearman coefficient method and there was a significant positive coefficient correlation between both RQ of Livin and YAPI gene expression, RQ of Livin gene expression with serum CEA in HCC group also between RQ of YAPI 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 YAPI 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 YAPI genes are associated with HCC and indicating bad prognosis of the disease. Previous findings were reported by other studies like Fan et al. who concluded that an increased expression of YAPI within PBMCs could serve as a bad indicator for the prognosis of HCC patients as that study reported high level of YAPI in mononuclear cells and showed positive linear correlation to Treg percentage which is immunosuppres-sant cells (Fan et al., 2017).

The CEA level, RQ of Livin and YAPI 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 YAPI gene expression can be considered as a bad sign for overall survival in HCC patients. This finding confirms what obtained by Zhang et al. (2017) who demonstrated that high level of YAP 1 together with low level of miRNA-345 was associated with low survival rate. Other study also reported that YAP-1 is associated with increased TGF-β (within HCC and hyperplasia of oval cells together with activation of inflammatory cell infiltration and fibrosis (Nishio et al., 2016).

HIC studies on human HCC samples showed that elevated expression of YAP correlates with poor tumor differentiation and is prognostic of bad outcome (Guo et al., 2015). YAP protein levels are upregulated starting from precancerous lesions, but overt nuclear localization of YAP can be found only in fully developed HCC and CC (Perra et al., 2014).

Livin gene expression was also reported as a bad prognostic marker by Augello et al., 2009 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.

Also, the study of Hua Guo et al., 2013 found that Livin protein expression was significantly higher in HCC tissues than that in normal hepatic tissues and hepatitis/hepatic cirrhosis tissues, with no significant

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**Table 5**

Relation between RQ of Livin gene and RQ of YAPI gene with tumor size by US in HCC group (n = 30).

| Tumor size by US | N | RQ of Livin gene | H | p  |
|-----------------|---|-----------------|---|----|
|                 |   | Min. – Max.     | Mean ± SD. | Median | 11.644^* | 0.003^* |
| ≤5              | 14| 3.54–9.93       | 7.13 ± 1.83| 7.01   |          |
| >5              | 9 | 5.67–20.80      | 11.37 ± 4.45| 11.53  |          |
| Multifocal      | 7 | 7.56–25.67      | 11.92 ± 6.18| 10.39  |          |

| Tumor size by US | N | RQ of YAPI gene | H | p  |
|-----------------|---|-----------------|---|----|
|                 |   | Min. – Max.     | Mean ± SD. | Median | 15.622^* | <0.001^* |
| ≤5              | 14| 4.75–11.89      | 6.94 ± 1.96| 6.45   |          |
| >5              | 9 | 6.25–36.89      | 18.35 ± 13.78| 13.78  |          |
| Multifocal      | 7 | 9.89–40.68      | 22.79 ± 24.67| 11.94  |          |

H: H for Kruskal Wallis test.
p: p value for comparing between the different categories. Statistically significant at p ≤ 0.05.

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**Fig. 4.** Relation between RQ of Livin and YAPI genes and tumor size in HCC group.
5. Conclusions

Based on the findings in our study, we concluded that there is overexpression of Livin and YAP1 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 path-physiological role in progress of HCC as well as in other cancer types in order to develop new therapeutic choices. However, there are some limitations in the current study; limited sample sizes together with that serum samples were not correlated with expressed levels of investigated gene expression, also IHC staining of livin and yap1 in the patient samples could help. A HCC group without HCV infection can be used as a reference group to confirm that the change of livin and YAP1 expression is due to HCC cancer alone or HCV or both.

Declarations

Author contribution statement

Eman Badr: Conceived and designed the experiments; Performed the experiments; Wrote the paper.

Ibrahim El Tantawy: Conceived and designed the experiments; Wrote the paper.

Mohamed Assar, Sahar Ali: Analyzed and interpreted the data; Wrote the paper.

Nehal Ibrahim: Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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