mir-1792 Expression In Various Stages Of Chronic Hepatitis C Viral (HCV) Infection: Correlation With PEG-10 And PTEN

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Abstract: **Background:** Circulating microRNAs (miRNAs) have been reported as a promising epigenetic biomarker. miRNAs have significant diagnostic potential for hepatocellular carcinoma (HCC). In this study, expression of miR-17-92 in Hepatitis (C)-related Liver Cirrhosis and HCC was evaluated. miR-17-92 expression was assessed using quantitative real-time Polymerase chain reaction (RT-PCR) in 145plasma samples [45 healthy controls, 25 HCC patients, and 75 cirrhotic patients with various Child-Pugh stages]. The relation between miR-17-92 and paternally expressed 10 (PEG10) and Phosphatase and tensin homolog (PTEN) proteins were investigated. **Results:** Our study found a significant reduction in miR-17-92 expression (P<0.001) in HCV-infected patients [either cirrhotic or HCC patients] when compared with the healthy control group. miR-17-92 expression levels with a cutoff value of 1.175-fold change had a 64% sensitivity and 34.9% specificity. With a cutoff value of 8.5 ng/ ml, alpha-fetoprotein (AFP) had a high sensitivity (87.5%) and specificity (100%) in the detection of HCC. BothPEG10 (P<0.001) and PTEN (P<0.01) were significantly decreased in HCC patients as compared with the healthy controls. The detection of miR-17-92, PEG10, and PTEN is valuable in elucidating the complex regulatory mechanisms governing HCC tumorigenesis. **Conclusions:** The combined detection of these factors may have clinical importance in prognostic, diagnosis, judgment, and therapeutic options for primary HCC.A future prospective large-scale study is recommended to support our findings and prove the mechanistic connections between miR-17-92 and PEG10/PTEN levels and HCV disease manifestations.

Kew Words: miR-17-92; HCC; PEG10; PTEN; Cirrhosis; HCV

1. Background

microRNAs (miR) are small non-coding RNA molecules; 18 to 22 nucleotide lengths. They play essential regulatory roles in apoptosis, cell proliferation, differentiation, angiogenesis, and, consequently, in carcinogenesis. By regulating protein-coding mRNAs mainly through translational inhibition or mRNA degradation, they mediate multiple signaling pathways. By imperfect base pairing with the 3-untranslated region (3-UTR) of target mRNAs, miRs could inhibit successful mRNA translation of target genes (Bartel, 2004; Alvarez-Garcia and Miska, 2005; Varnholt et al., 2008; Giordano et al., 2013). In the nucleus, biogenesis of miRis initiated by RNA polymerase II/III to create a primary transcript (pri-miRNA) which then cleaved by RNase III enzyme Drosha and DGCR8/Pasha excising the stem-loop to form the pre-miRNA, exported to the cytoplasm for final processing into mature miRNA (Winter et al., 2009). In the liver, miR-17-92 members are present at the center of cellular pathways with assumed targets for genes involved in lipid metabolism and metabolic function (Xie et al., 2009; Rottiers et al., 2012), inflammatory and innate immune responses (Ji et al., 2011), cell cycle control and proliferation (Mendell, 2008; Dakhlallah et al., 2013), cell differentiation (Sun et al., 2013), epithelial-mesenchymal transition (Liu et al., 20011), and cell death (Tsitsiouand Lindsay, 2009). Some studies recorded that miR17-92 plays an oncogenic role and provides cisplatin chemo-resistances in human prostate cancer cells (Conv et al., 2015). In the 17-92 cluster, miRNAs can target phosphatase and tensin homolog (PTEN) transcripts and E2F1 genes (Pickering et al., 2009; Gruszka et al., 2018). PTEN is a crucial adverse regulator of the highly oncogenic pathway (von-Haaften and Agami, 2010). PTEN mutation is a regular event in advanced stages of various human malignancies (Hu et al., 2003). The role of miR17-92 in controlling the transcription factor E2F expression, which participates in cell proliferation through controlling the transcription of various cell cycle key components and promoting tumor angiogenesis, is previously reported (Olive et al., 2010; Li et al., 2014; Li et al., 2017). Moreover, E2F was likely a key transcriptional factor responsible for regulating paternally expressed gene 10 (PEG10) (Wang et al., 2008). It has been proposed that PEG10 is derived from a retro-transposon, which is previously integrated into the mammalian genome (Ono et al., 2001). PEG10 is maternally silenced and paternally expressed. An increased expression of PEG10 in human HCC and mouse liver regeneration suggests that this gene has growth-promoting activity (Tsou et al., 2003; Li et al., 2006; 2016). It has different expression patterns in various types of cancer, including leukemia, pancreas, breast, prostate, gallbladder cancers, and HCC (Li et al., 2006). To date, scarce information is available regarding the cluster miRNA 17-92 and HCV (Lorini et al., 2020). Thus, this study aims to evaluate the miR-17-92 cluster expression in HCV-infected and HCC patients and its influence on PEG 10 and PTEN expression.

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2. Methods

2.1. Clinical Data

The local ethical committee approved this study at the National Liver Institute. In compliance with their Health and Human Ethical Clearance Committee recommendations for clinical trials, all investigations were carried out following the National Liver Institute, Menofiya University. Each subject provided signed informed consent. One hundred and forty-five participants were involved in this work. One hundred HCV-infected patients were divided into 25 cases with HCC and 75 with cirrhotic liver categorized into three stages of liver cirrhosis according to the Child-Pugh score (Pinter et al., 2016);25 cases from Child-Pugh class "A,"25 cases from class "B" and 25 cases from class "C" patients. In parallel, 45 healthy controls tested negative for HBV, HCV, and HIV infection were enrolled in this study. They have normal liver function activities and no history of alcohol abuse or liver disease.

2.2. Eligibility criteria

HCV infected patients were divided according to their age range, histological proof of HCC, absence of major organ dysfunction, Tri Phasic CT Abdomen of the hepatic focal lesion, and serum AFP. Serum bilirubin for HCC cases was not more than 3 mg/dl and more than 10 mg/dl for cirrhotic patients.

2.3. Liver function tests

Serum alanine and aspartate aminotransferase (ALT and AST) were estimated (Reitman and Frankel, 1957). Serum alkaline phosphatase (ALP)and total serum protein (TP) were measured colorimetric according to the method of Kind and King (1954) and Jacobs et al. (1964), respectively. The serum albumin level (Alb) was measured using the technique of Baure et al. (1982). Meanwhile, serum bilirubin concentration [total and direct] was calculated spectrophotometrically using the colorimetric (Diazo) modified method (Doumas et al., 1985). All done by Spectrum Diagnostics reagent kits.

2.4. Serum Alpha-Fetoprotein Level

AFP level was measured in serum using sandwich solid-phase enzyme-linked immunosorbent assay (ELISA) (BioVendor – Laboratorni medicina, Czech Republic) according to the methods of Uotila et al. (1981).

2.5. miR-17-92 detection by Real Time-PCR Analysisand reverse transcription reaction [qRT-PCR]

Total human miR was extracted using a Qiazol RNA isolation kit RNeasy Mini Kit [cat. No. 217004, QIAGEN, Germany] to study the expression of miR-17-92 cluster. Using the miSCRIPT reverse transcription kit, extracted RNA was reverse-transcribed to cDNA; then real-time qPCR was performed using the miSCRIPT Syber Green PCR kit (QIAGEN, cat. no.218073). The miR-17-92 cluster forward primer is 5′-CAGTAAAGGTAAGGAGAGCTCAATCTG-3′, reverse primer is 5′-CATACAACCACTAAGCTAAAGAATAATCTGA-3′ (Zhu et al., 2015). As an internal control, the β-actin gene was quantified in parallel [primer forward: 5'-GAGACCTTCAACACCCCAGCC-3' and primer reverse: 5'-GGATCTTCATGAGGTAGTCAG-3' (Li et al., 2016). miRNA expression was normalized to their corresponding internal control genes and relative change was calculated using the $2^{-\Delta\Delta}$CT method as previously described by Omran et al. (2020).

2.6. PTEN and PEG10 determination

Following the manufacturer's instruction, human PTEN and PEG10expression levels were determined in serum by ELISA kit Bioneava, Co., Ltd., China]. Color intensity was calculated as the optical density (OD) value measured at 450 nm using Bio-Rad 4100, and the results were expressed as pg/ml.

2.7. Statistical analysis

All statistical analyses were conducted using version 19 of the Statistical Kit for Social Science (SPSS) (LEDA Technology Inc). The mean with corresponding standard deviation (Mean ± SD) is viewed in clinical data. To compare various groups, one-way variance analysis (ANOVA) was used, followed by the Tukey test as a post-hoc test. Using the Chi-square measure, the frequency was compared. To define the optimal sensitivity and specificity of the miRNA-17-92 expressions, receiver operating characteristic [ROC] curves were constructed. The level of significance was $P<0.05$.

3. Results

3.1. Patient characteristics

HCV-infected patients [125 subjects] were enrolled in this study (Supplementary Table 1). Twenty-five patients were diagnosed with HCC (22 males and 3 females with a mean age of 56.2±5.8 years). The mean age of cirrhotic patients was 56.0±12.2 years in the whole cohort, 49.0±13.7 in Child A, 54.5±9.1 in Child B, and 64.6±7.7 years in Child C groups. Cases suffering from HCC have a mean nodules number of 3.1±1.3 and a mean tumor size of 6.8±2.8 cm. Forty-five healthy controls with a mean age of 48.3±6.9 years were run parallel.

3.2. Clinicopathological characteristics of HCC patients

As shown in Table (1), HCC patients produced a wide range of AFP values (from normal to 2355 ng/ml) with a mean value of1288.7±3492.1 ng/ml. In 40% of HCC patients, hepatic focal lesions were single with regard to CT imaging, 72% originating from the right lobe percent, while 44% were single with a size between 3-6 cm.

3.3. Expression of plasma miR-17-92 level in different groups

Figure (1) illustrates miR-17-92 expression in all studied groups. Our results showed that miR-17-92is down-regulated in cirrhotic patients with Child A($P<0.01$), Child B($P<0.01$), and Child C ($P<0.001$) cirrhotic patients as well as in HCC ($P<0.01$) patients when compared with that of the control group. miR-17-92 is up-regulated in cirrhotic patients with Child A and B compared to HCC patients, although the elevation is statistically insignificant.

3.4. Expression of serum PEG10 level in different groups

Figure (2) shows that serum PEG10 in HCC patients was significantly reduced (P<0.001) as compared with healthy controls. An insignificant change was observed when patients at various cirrhotic stages are compared with healthy controls. A significant increase of PEG10 level in cirrhotic patients was reported with a maximum elevation of PEG10 in Child A and B(P<0.001) cirrhotic groups than HCC patients.

3.5. Differential expression of serum PTEN levels

In HCC patients, PTEN serum level was decreased significantly (P<0.01) when compared with healthy controls (Figure 3). In contrast, cirrhotic patients exhibit an insignificant change in PTEN expression in relation to normal cases. Contrastingly, the PTEN level was increased significantly(P<0.05) in Child A cirrhotic patients only compared with HCC patients.

3.6. Receiver operating characteristic (ROC)Curve

ROC curves were performed to estimate the best cutoff for improving the specificity and sensitivity of studied biomarkers. As shown in figure(4a), the area under the curve (AUS) for miR-17-92, was 0.347witha sensitivity of 64% and specificity of 34.9% with 95% confidence interval (CI):0.218-0.477] at 1.175-fold change. Meanwhile, AUC for AFP (Figure 4b)was 0.928 with 95% CI: 0.492-0.761. At 8.5 ng/ml cutoff value, the sensitivity was 87.5%, while the specificity was 100% (CI: 0.842-1).

3.7. Correlation between miR-17-92, PEG10 and PTEN in HCC patients

A significant positive correlation between PEG10 and PTEN in the HCC group was observed (r=0.402, P<0.05). Also, PTEN is positively correlated with miR-17-92 (r=0.505, P<0.01).

4. Discussion

Our data showed that HCC is often observed in males than females. This observation is almost in all parts of the world. A noteworthy aspect of HCC is that men have a higher prevalence and worse projection in low and high incidence regions than women (Chen et al., 2016; Torre et al., 2016; Siegel et al., 2017). Increasing evidence has shown that sex inconsistently might be mediated by particular androgens' stimulatory effects and the defending impacts of estrogen in HCC development and progression. The sex imbalance in HCC may be associated with the ratio of estrogen and testosterone, indicating that active signaling pathways mediated by estrogen and androgen could influence the initiation and development of HCC. (Naugler et al., 2007; Wang et al., 2009; Yang et al., 2012). Thus, estrogen reduces the frequency and metastasis of hepatocarcinogenesis. Li et al. (2019) summarized the recent research reconnoitering the sex hormones/chromosomes implication in this process. They pointed to two tumor-promoting and inhibiting axes which involved sex hormones and their receptors across separate pathways. Thus, sex chromosome genetic modifications could cause the underlying mechanism of the sex discrepancy in HCC. Identifying circulated miRNA profiles specific for HCC is an emerging area of particular interest. miRNAs have been embroiled in cancer. Increasing pieces of evidence are referring to the leading role of miRNAs in the progression of cancer. They indicated that by controlling several cellular pathways, miRNAs could act either as tumor suppressors or oncogenes' oncomirs' (Wang et al., 2012; Duyu et al., 2014; Liu et a., 2014). Our findings showed that in HCV-related cirrhotic and HCC patients, miR17-92 is differentially expressed than the control group. In the plasma of HCC patients, the amount of miR17-92 is substantially reduced. Our result agreed with Tanaka et al. (2009), who reported that miR-17-92 is reduced in acute leukemia patients' plasma. Our data is supported by previous studies' findings, which said that the miR-17-92 cluster was down-regulated in HCC patients (Petrocca et al., 2008; Cardin et al., 2012). Liu et al. (2020) demonstrated that miR-17-5p level was down-regulated in HCCs, specially in patients with postoperative metastasis, and these data provide evidences about the potential role for miR-17-5p in HCC progression. On the contrary, the data of Tan et al. (2014) and Zhu et al. (2015) confirmed that the miR17-92 cluster is thoroughly expressed in patients with HCC. Although, the expression of miR 17-92in their studies were investigated in the HCC cell lines. The present study's data found a significant reduction in PEG10 level in HCC patients. This finding parallels Shyu et al. (2016) results, who documented hat PEG10 expression is diminished in HCC tissue. No previous data have been reported about the level of PEG10 in the circulation of HCC patients. Otherwise, earlier studies of Wang et al. (2008) and Saad et al. (2013) said that PEG10 expression was significantly elevated in HCC patients than healthy control patients PEG10 was differentially expressed in various human malignancies such as breast, prostate, pancreas, gallbladder cancers, leukemia and HCC (Li et al., 2006). Our results illustrated that the PTEN serum level was decreased significantly in HCC patients compared with healthy controls. Simultaneously, PTEN level was markedly increased in Child A patients than HCC patients. This finding agrees with Ruan et al. (2012), who reported that the over expression of PTEN is associated with improving the therapeutic option of HCC. A related outcome is observed by Khalid et al. (2017), who proved that the expression of PTEN is reduced in HCC patients. E2F-1 is one of the E2F family transcriptional factors (Uhlén et al., 2005), is a critical transcriptional factor participating in PEG10 regulation (Wang et al., 2008). It could bind to the PEG10 promoter, and, in E2F-1 knockdown or over expression, the binding efficiency was reduced or increased, respectively. The same results were observed in tumor cells at protein levels. E2F1knockdown cells showed a reduction inPEG10 mRNA, with a concomitant decrease in PEG10 protein (Hofmann et al., 1969; Campanero and Flemington, 1997; Peng et al., 2017). miR17-92 plays an essential role in controlling E2F expression (Tanaka et al., 2009; Olive et al., 2010). It can inhibit E2F1, which consequently leads to down regulation of PEG10.Moreover, miR17-92 inhibits E2F1 and reduces PTEN expression (Ernst et al., 2009).By its attachment to the promoter region of the E2F1 transcriptional factor, PTEN participates in the regulation of E2F1 expression (Malaney et al., 2018).

5. Conclusions

In conclusion, we have shown that miR-17-92 is down-regulated in most HCC and liver cirrhosis cases. A severe reduction of miR-17-92, PEG10, and PTEN are characteristic of HCC. The detection of their circulating level is valuable in explaining the complexity of the regulatory mechanisms controlling HCC tumorigenesis. Moreover, the integrated detection of these parameters may have clinical significance in prognostic, diagnosis, verdict, and therapeutic options for primary HCC. Exploring the real mechanism that involved the 3 selected proteins could be further improved by measuring the expression of the E2F1 transcription factor.

6. Abbreviations:

HCV: Hepatitis C virus; miRNAs: microRNAs; PEG10: paternally expressed 10; PCR: Polymerase Chain Reaction; CBC: Complete blood count; AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; Alb: Albumin; EDTA: Ethylene Diamine Tetra-Acetic Acid; LD: Linkage Disequilibrium; PTEN: Phosphatase and tensin homolog; HCC: hepatocellular carcinoma; AFP: fetoprotein; ELISA: enzyme-linked immunosorbent assay

7. Declarations

Ethics approval and consent to participate: In accordance with the National Liver Institute (NLI), the ethical committee of Menoufia University, all investigations were carried out reference number is not applicable. Written Informed consent was taken from each participant.

Consent for publication: Not applicable.

Availability of Data and Materials: Provided by corresponding author on request

Competing Interest: The authors declare that no conflicts of interest

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Author contribution: This work was carried out in collaboration between all authors: RT designed the research; AE and AE, provided patients; HZ performed the experiment; YA, analyzed and interpreted the data. RT and HZ wrote the manuscript. All authors revised, and approved the final manuscript.

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9. Figure legends

Figure 1: Relative fold change of miR-17-92 expression in different groups. (*): Significant difference from HCC group (** P<0.01 and ***P< 0.001)

Figure 2: Differential expression of serum PEG10 level in different groups (*): Difference from control group (***P<0.001); (▪): Difference from HCC group (▪▪▪P<0.001); (•): Difference from Child A group (•P<0.05)

Figure 3: Differential expression of serum PTEN levels (pg/ml) in different groups. Data are presented as mean ± SE. (*): Difference from the control group (***P<0.001); (▪): Difference from HCC group (▪P<0.05)

Figure 4a: ROC Curve analysis showing the diagnostic performance of miR-17-92expression in blood for discriminating patients with HCC from non-HCC patient

Figure 4b: ROC Curve analysis showing the diagnostic performance of AFP expression in serum for discriminating patients with HCC from the non-HCC patient.

Table (1): *Clinicopathological characteristics of HCC patients*

Parameter	Number [%]
AFP level	
Up to 10	3 [12%]
< 20 ng/ml	4 [16%]
20-100 ng/ml	6 [24%]
>100-400 ng/ml	2 [8%]
>400-1000 ng/ml	2 [8%]
> 1000 ng/ml	8 [32%]
Number of focal lesions	
Single	10 [40%]
Multiple	15 [60%]
Site of focal lesions	
Right lobe	18 [72%]
Left lobe	7 [28%]
Tumor size by CT	
<3 cm	14 [56%]
3–6 cm	11 [44%]

Figure (1)

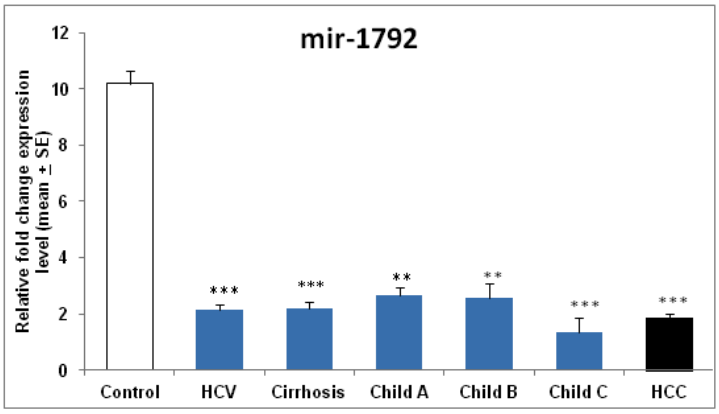


Figure (2)

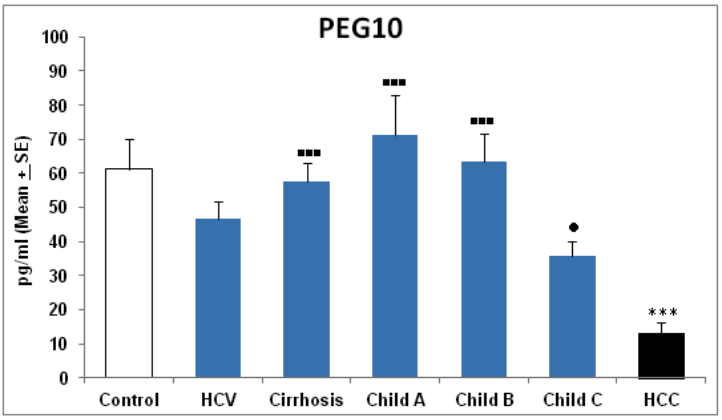


Figure (3)

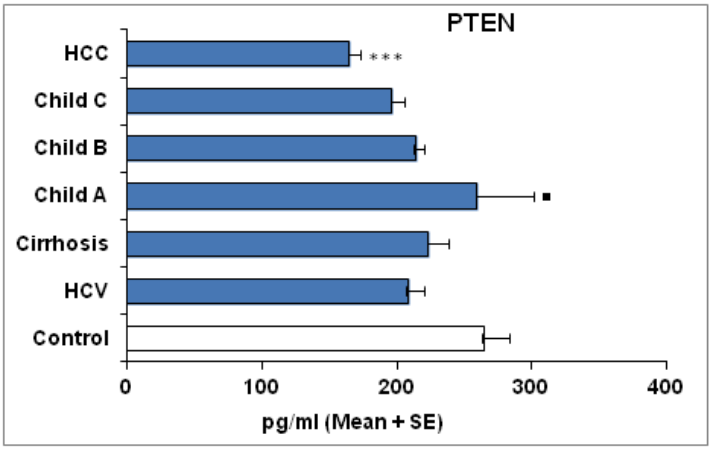
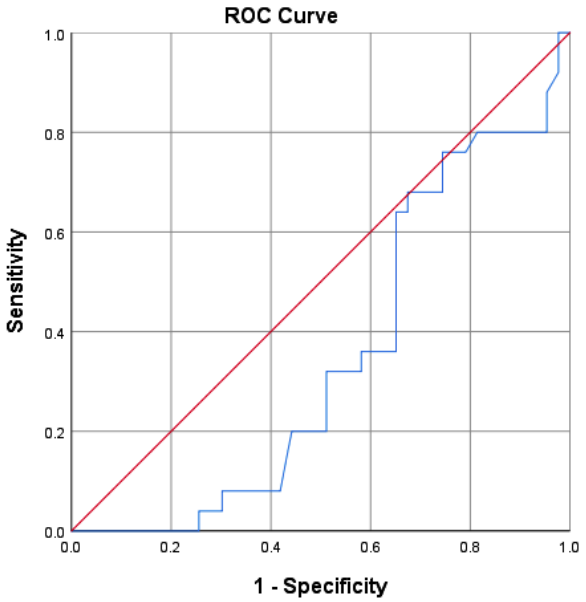
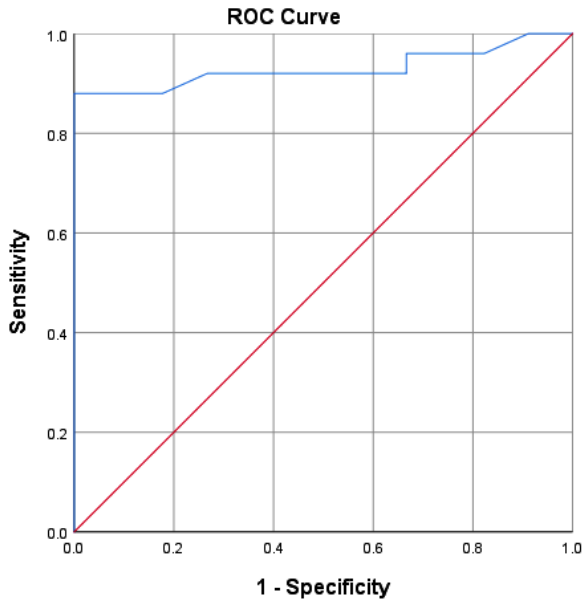


Figure (4)





Supplementary Table (1): *Demographic and biochemical data of studied groups*

Parameter	Control n=45	Patients with cirrhosis				HCC n=25	*P*
		Child A n=25	Child B n=25	Child C n=25	Total n=75		
Age	48.3±6.9	49.0±13.7	54.5±9.1	64.6±7.7	56.0±12.2	56.2±5.8	NS
Sex (M: F)	43:2	20:5	16:9	22:3	58:17	22:3	<0.05
ALT (U/L)	17.1±5.8	24.7±15.7	53.9±68.4	49.4±45.5	42.7±49.3	54.3±28.4	<0.001
AST (U/L)	20.9±6.1	25.4±17.1	47.3±36.7	87.4±89.2	53.4±61.5	67.7±40.1	<0.001
Alb (g/dl)	4.3±0.5	4.3±0.52	3.1±0.5	2.3±0.4	3.2±0.9	3.4±0.7	<0.001
TP (g/dl)	9.9±14.4	7.8±0.8	6.3±0.9	5.6±1.1	6.58±1.3	12.1±18.4	NS
TBil (mg/dl)	0.6±0.3	0.6±0.3	4.0±6.3	7.1±7.3	3.9±6.1	1.32±0.7	<0.001
DBil (mg/dl)	0.1±0.01	0.2±0.1	3.0±5.4	5.6±6.6	2.9±5.3	0.51±0.5	<0.001
INR (%)	1±0.1	1.1±0.08	1.4±0.2	1.6±0.4	1.3±0.3	1.17±0.1	<0.001

All data are presented as mean ± standard Error (mean ± SE). NS = not significant. Aspartate aminotransferase (AST); Alanine aminotransferase (ALT); Albumin (Alb), Total protein (TP), Total (TBil) and Direct (DBil) bilirubin, international normalized ratio (INR)