Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2021 Apr 29; 9(A):222-228.
https://doi.org/10.3889/oamjms.2021.5909
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Biochemistry*

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Insulin-like Growth Factor Initiates Hepatocellular Carcinoma in Chronic Hepatitis C Virus Patients through Induction of Long Non-coding Ribonucleic Acids AF085935

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Edited by: Slavica Hristomanova-Mitkovska
Citation: Mostafa A, Ibrahim NE, Sabry D, Fathy W, Elkazaz AY. Insulin-like Growth Factor Initiates Hepatocellular Carcinoma in Chronic Hepatitis C Virus Patients through Induction of Long Non-coding Ribonucleic Acids AF085935. Open Access Maced J Med Sci. 2021 Apr 29; 9(A):222-228. https://doi.org/10.3889/oamjms.2021.5909
Keywords: Hepatitis C virus; Hepatocellular carcinoma; Insulin-like growth factor; Forkhead box class O and long non-coding ribonucleic acid AF085935
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E-mail: nohaelsayed855@gmail.com
Received: 19-Feb-2021
Revised: 12-Mar-2021
Accepted: 29-Mar-2021
Copyright: © 2021 Abeer Mostafa, Noha E. Ibrahim, Dina Sabry, Wael Fathy, Amany Y. Elkazaz
Funding: This research did not receive any financial support
Competing Interests: The authors have declared that no competing interest exists
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Abstract

BACKGROUND: Hepatitis C virus (HCV) is the most commonly occurring hepatic infection worldwide. Chronic HCV infection usually complicated with cirrhosis and even hepatocellular carcinoma (HCC) with significant morbidity and mortality.

AIM: The aim of this study was to clarify the molecular mechanism by which HCV can induce HCC and identify a new diagnostic marker for early detection of HCC.

METHODS: A total of 180 participating subject were divided into three groups: Group 1: 60 healthy individuals (controls), Group 2: 60 HCV-infected patients, and Group 3: 60 HCV patients developed HCC. Serum insulin-like growth factor (IGF), Forkhead box class O (FOXO), and long non-coding ribonucleic acid (LncRNA) AF085935 were evaluated.

RESULTS: Serum IGF was significantly elevated in HCV and HCC patients, while FOXO and LncRNA AF085935 were significantly upregulated in HCC. IGF significantly correlated with and LncRNA AF085935.

CONCLUSION: HCV can induce IGF with subsequent induction of LncRNA AF085935 and FOXO.

Introduction

Hepatocellular carcinoma (HCC) is a major cause of liver-related death worldwide. Hepatitis C virus (HCV) infection is the most important cause of hepatic cirrhosis, which increases the risk for HCC [1]. HCC as a result of HCV is a stepwise process taking long time over 20–40 years. The core protein of HCV can induce lipogenesis and disturb the oxidative stress metabolism [2].

HCV proteins inhibit the tumor suppressor genes and cell cycle check points and activate cellular growth and division. The genes of HCV non-structural protein also induce fibrosis and the HCC development through inducing transforming growth factor (TGF)-beta and stimulating hepatic stellate cells [3].

Human Forkhead box class O (FOXO) transcription factor is activated in response to a wide range of external stimuli, such as insulin, growth factors, nutrient levels, and oxidative stress. FOXO regulates the expression of genes that control the metabolic processes, furthermore, it has been involved in tumor suppression by upregulation of apoptotic genes and those responsible for cell cycle arrest. Thus, it seems that FOXO provides new insights into the molecular pathogeneses of HCC and provides opportunities for developing new antitumor drugs [4].

Fox proteins activity is regulated by multiple post-translational modifications such as phosphorylation, acetylation, and ubiquitylation. In the nucleus, Fox proteins are active and act as transcriptional regulators. However, in the cytoplasm, Fox proteins are inactive and exposed to proteasomal degradation. Phosphorylation and acetylation control the import and export of FOXO from the nucleus [5].

The liver cells utilize FOXO to respond to the stresses induced by disease, in addition to regulation of metabolic pathways such as gluconeogenesis. Thus, FOXO3 is essential for antioxidant responses and

autophagy and is affected in hepatitis C infection and fatty liver [6].

FOXO is negatively regulated by the PI3K-PKB/AKT signaling pathway and has been considered to be tumor suppressors. FOXO inhibits tumor cell establishment in addition cancer cell growth and survival [7]. However, recent studies reported that tumor cells can utilize FOXO for its own to withstand adverse conditions, thus tumor progression and metastasis [8].

Insulin-like growth factor (IGF-1) is a secretory protein consisting of a single polypeptide chain with 70 amino acids, which reveals ~50% sequence identity to that of insulin. The signaling of IGF has an important autocrine, paracrine, and endocrine role [9]. IGF family and their receptors have an essential role in the progress of tumors [9]. Recent reports have indicated that IGF facilitates the progression of cancer by regulating cell proliferation, angiogenesis, immune evasion, and metastasis [10].

HCV proteins have been involved in control of cell cycle, through their interaction with control proteins of the cell cycle as p21, p53, cyclins, proliferating cell nuclear antigen, proto-oncogenes (c-fos, c-jun), and growth factors/cytokines, for example, tumor necrosis factor (TNF)-α, TGF-β and their receptors, transcription factors nuclear factor-kappa B, and proteins of apoptosis [11]. HCV core acts as a positive regulator of IGF transcription through the protein kinase C pathway that stimulates cell proliferation and division, thus IGF plays an important role in hepatitis C-induced formation of HCC [12].

Long non-coding ribonucleic acids (LncRNAs) are non-coding RNAs, shorter than 200 nucleotides in length [13]. LncRNAs have important roles in several biological processes, through multiple regulatory processes such as chromatin remodeling [14], protein inhibition, transcriptional coactivation or corepression, and post-transcriptional modification [15]. LncRNAs have been used as a screening marker of multiple cancers [16].

LncRNAs have tissue-specific expression and their expression is regulated in correlation with cell cycle, survival, and immune response regulatory genes. Moreover, several LncRNAs are transcriptionally regulated by tumor suppressor genes or oncogenes [17]. Thus, in this study, we aim to detect the underlying mechanism by which HCV could be complicated by HCC, for early prediction of HCC in chronic HCV patients.

Materials and Methods

Study subjects

The study was conducted on 180 subjects matched sex and age, the patients were enrolled from the tropical department, Faculty of Medicine, Cairo University. Sample size was calculated using the G power software. Based on Brabant and Wallaschofski, 2007, [9] we found that 60 participants per group were appropriate sample size for the study with total sample size 180 participants (three groups). The power is 80% and α error probability = 0.05, effect size f = 0.234.

The groups were divided as the following: Group 1: 60 healthy individuals (controls), Group 2: 60 HCV-infected patients, and Group 3: 60 HCV patients developed HCC. Group 1: Sixty healthy individuals served as healthy controls. All of them were free of apparent diseases, disorders, or infection. They were negative for hepatitis B surface antigen (HBsAg), hepatitis B (HB) core Ab, HB virus, deoxyribonucleic acid (DNA), HCV Ab, HCV ribonucleic acid (RNA), and any liver involvement as documented by an abdominal ultrasound examination. None of them had a history of liver insult, viral, metabolic, or drug exposure Group 2: Sixty HCV-infected patients fulfilled the inclusion criteria included elevated serum levels of alanine aminotransferase (ALT) and aspartate transaminase (AST) within 6 months prior to entry the study, positive HCV antibodies, detectable HCV-RNA, HCV genotype 4, and liver biopsy showing histological evidence of chronic hepatitis and they were never previously treated with interferon. Group 3: Sixty HCV patients developed HCC patients that presented with liver cirrhosis or HCC, fulfilled the inclusion criteria included hemoglobin (<11 g/dL), total leukocytes count (<3000/mm^3), neutrophil (<1500/mm^3), platelets (<100,000/mm^3), presence of antinuclear antibodies (ANA titer <1/160), HBsAg seropositivity or autoimmune hepatitis or coinfection with the human immunodeficiency virus, or the presence of any chronic systemic illness were excluded from this study(exclusion criteria).

All HCC patients were diagnosed as HCC according to clinical examination, radiological investigations (including unequivocal clinical and imaging data, ultrasonic computed tomography scanning), histological diagnosis (determined by means of percutaneous biopsy), and laboratory investigations (total and direct bilirubin, transaminases, alkaline phosphatase (ALP), albumin, prothrombin time and concentration, and alpha fetoprotein [AFP] levels). All HCC patients were classified as Class A CHILD score.

RNA extraction from whole blood

After obtaining patients consent, total RNA was extracted from whole blood of all studied groups with RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Quantitation and purity assessment for RNA samples were done using the

NanoDrop® (ND)-1000 spectrophotometer (Nano Drop Technologies, Inc., Wilmington, USA).

Real-time quantitative polymerase chain reaction (qPCR)

SensiFAST™ SYBR® Hi-ROX One-Step Kit, catalog no.PI-50217 V had been formulated for highly reproducible first-strand cDNA synthesis and subsequent real-time PCR in a single tube in a 48-well plate using the StepOne instrument (Applied Biosystem, USA). The PCR run was performed on a Rotor Gene Real-Time PCR System (Qiagen). Normalization for variation in the expression of each target gene was performed referring to the mean critical threshold (CT) values of ß-actin housekeeping gene expression by the ΔΔCt method [18]. Primers sequence for all studied genes was listed as following in Table 1.

Table 1: Primer sequence of all studied genes

Gene	Forward (5'-3')	Reverse (5'-3')	References
FOXO	5′-TGCATCCATGGACAAC AACA-3′	5′-CGAGGGCGAAATG TACTCCAGTT-3′	[19]
Lnc-RNA AF085935	5′-CAGGGCAGCAAGGTG TTTTC-3′	5′-TTGGTGGGTTGCC TGATACC-3′	[20]
ß actin	5′-GGCGGCACCACCATG TACCCT-3′	5′-AGGGGCCGGACTC GTCATACT-3′	[20]

FOXO: Forkhead box class O, Lnc-RNA: Long non-coding ribonucleic acid.

Estimation of serum level of IGF

The serum level of IGF of all studied groups was estimated following the instruction of IGF ELISA kit catalog n. OKEH00080.

Estimation of serum AFP level by enzyme-linked immunosorbent assay (ELISA)

The serum level of AFP of all studied groups was estimated following the instruction AFP ELISA kit which was provided by Immunospec Corporation, Canoga Park, USA, Catalog No.C29-332.

Statistical analyses

Data were analyzed using SPSS version 22. Data are presented as mean and standard deviation. One-way ANOVA with multiple comparison *post hoc* test was used for comparing between three groups. Qualitative data were compared using the χ^2 test. A difference between groups was considered to be significant if $p \leq 0.05$. Correlation between quantitative variable was done using Pearson correlation. Receiver operating characteristic (ROC) curve was constructed with area under curve (AUC) analysis performed to detect best cutoff value of AFP, IGF, FOXO, and Lnc-RNA AF085935 for the detection of HCC, multivariate regression analysis was done to detect the best predictor of HCC among studied parameters.

Results

Clinical characteristics of all studied groups

The clinical and laboratory data of all studied groups are shown in Table 2. The statistical analyses of chosen factors revealed that there were very highly significant differences between the HCV group and HCC group when compared with healthy individuals (control group) in total bilirubin level, ALT, AST, and ALP, furthermore, total bilirubin level, direct bilirubin, and ALP were significantly higher in HCC than HCV patients. Albumin was significantly lower in HCV and HCC patients than in control group and significantly lower in HCC than HCV patients. While no significant difference among three studied groups regarding age of patients and creatinine levels (p value 0.05) . On the other hand, PC percentage was significantly higher in control group than in HCC group and in HCV in compare to HCC. The levels of AFP in HCC patients were significantly higher than control group and HCV group ($p < 0.001$).

Table 2: Clinical characteristics of all studied groups

Parameters	Control Group (I) n=60	HCV Group (II) n=60	HCC Group (III) n=60	p-value
Age (years)	48.10 ± 11.40	49.89 ± 9.70	52.91 ± 7.27	> 0.05
T-bilirubin (mg/dl)	0.88 ± 0.23	0.84 ± 0.37	1.58 ± 1.22*#	< 0.001
D-bilirubin (mg/dl)	0.23 ± 0.12	0.24 ± 0.16	0.68 ± 0.64*#	< 0.001
AST (U/L)	28.13 ± 6.83	69.64 ± 14.68*	64.78 ± 39.92*	< 0.001
ALT (U/L)	27.15 ± 7.56	68.33 ± 42.56*	61.6 ± 42.42*	< 0.001
ALP (U/L)	66.9 ± 29.33	88.8 ± 34.9	155 ± 97.38*#	< 0.001
Albumin (g/dl)	4.17 ± 0.52	3.82 ± 0.51*	3.31 ± 0.61*#	< 0.001
Creatinine (mg/dl)	0.94 ± 0.31	0.96 ± 0.28	1.86 ± 0.18	> 0.05
PC (%)	91.83 ± 5.77	88.1 ± 7.33	77.98 ± 14.37*#	< 0.001
AFP (ng/ml)	4.1 ± 2.27	12.88 ± 6.22	3420.05 ± 1474.36*#	< 0.001

(*): Denotes significant difference in comparison to control group, (#): Denotes significant difference in comparison to HCV group, AST: Aspartate transaminase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, AFP: Alpha-fetoprotein.

FOX-O, LncRNA AF085935 upregulated in HCC but not HCV

By comparing the amplification frequency between controls and HCV patients and HCC patients, there was a significant increase in FOXO, LncRNA AF085935 expression in HCC patients compared to HCV and control groups ($p < 0.001$). But no significant difference in their expression between HCC and HCV patients (p value 0.05) (Figure 1a and b).

IGF significantly elevated in HCV and HCC patients

The serum IGF-II was significantly higher in HCV and HCC patients compared to the healthy control ($p < 0.001$), in addition, its level was significantly higher in HCC patients than HCV (Figure 1c).

IGF significantly correlated with LncRNA AF085935 in HCC patients

Correlation analysis between studied parameters revealed that IGF-II significantly positively

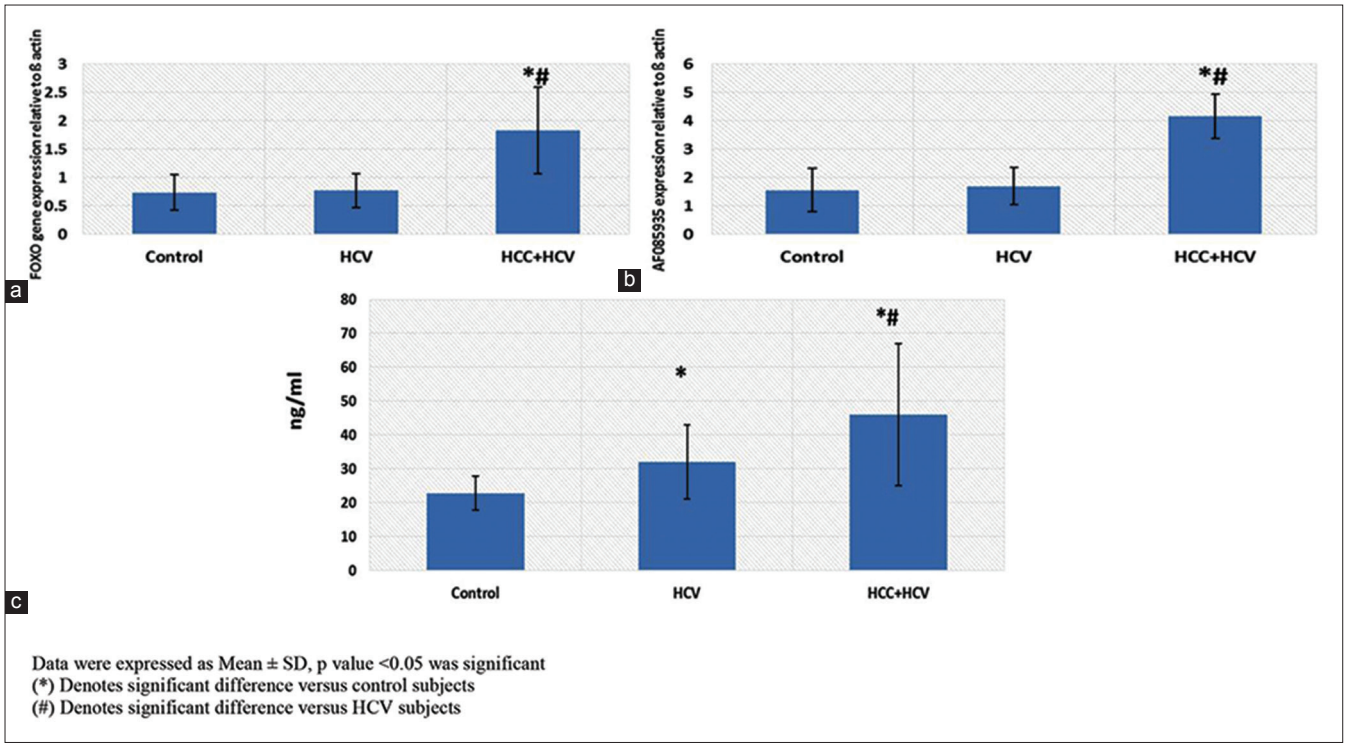

Figure 1: (a and b) Diagrammatic representation of significant increase in Forkhead box class O, long non-coding ribonucleic acid AF085935 expression in hepatocellular carcinoma (HCC) patients compared to hepatitis C virus (HCV) and control groups (p < 0.001). But no significant difference in their expression between HCC and HCV patients. (c) Diagrammatic representation of the serum level of insulin-like growth factor-II protein in in HCC patients compared to HCV and control groups (p < 0.001)

correlated with LncRNA AF085935 in HCC patients (r = 0.253, p < 0.001), furthermore, LncRNA AF085935 is significantly positively correlated with FOXO (r = 0.202, p = 0.003) (Table 3).

Table 3: Correlation analysis between studied parameters in HCC patients

Variables	IGF	FOXO	LncRNA
IGF			
r	1	0.064	0.253**
p-value		0.351	0.000
FOXO			
r	0.064	1	0.202**
p-value	0.351		0.003
LncRNA			
r	0.253**	0.202**	1
p-value	0.000	0.003	

**: Correlation is significant at the 0.01 level (two tailed), IGF: Insulin-like growth factor, FOXO: Forkhead box class O, LncRNA: Long non-coding ribonucleic acid.

FOXO, IGF, and LncRNAs AF085935 are good diagnostic marker for HCC

ROC analysis for all studied parameters revealed that they can be considered good diagnostic markers for HCC. LncRNA AF085935 is the best diagnostic marker among them. ROC curve for IGF serum levels showed that the best chosen cutoff level was 32.3 ng /ml, at which the sensitivity was 71% and the specificity was 70%. The AUC was 0.755. The ROC curve for FOXO gene expression levels showed that the best chosen cutoff level was 0.99, at which the sensitivity was 81% and the specificity was 82%. The AUC was 0.91. The ROC curve for LncRNA AF085935 showed the best chosen cutoff level was 3.2, at which the sensitivity was 100% and the specificity was 90%. The AUC was 0.966. The ROC curve for AFP showed the best chosen cutoff level was 26.9, at which the sensitivity was 71.1% and the specificity was 98.6%. The AUC was 0.791 (Figure 2 and Table 4).

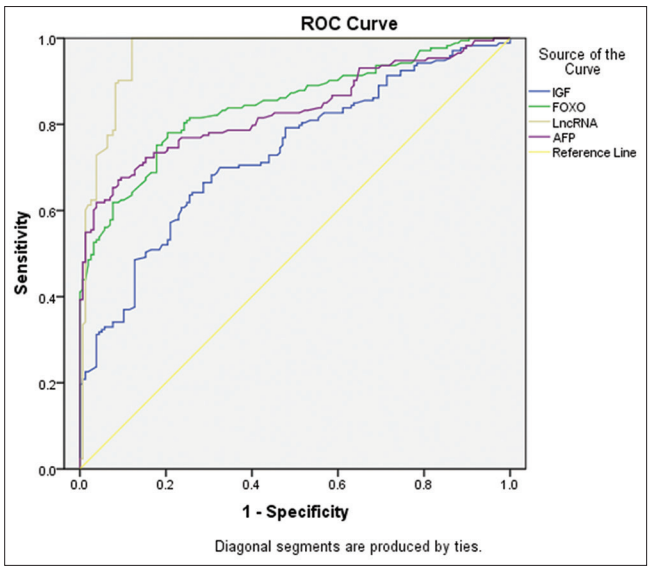

Figure 2: Receiver operating characteristic analysis of studied genes

Multivariate regression analysis for prediction of occurrence of HCC on HCV patients

Regression analysis for the detection of the risk factors for HCC development on top of chronic HCV infection revealed that LncRNA AF085935 represents

the most significant risk factor for HCC (p < 0.001) odds ratio = 18.824, CI (8.196–43.234). While the odds ratio for other studied parameters; FOXO = 6.146, CI (2.390–15.806), for IGF the odds ratio = 1.044, CI (1.012–1.076), for AFP the odds ratio = 4.08, CI (1.07–15.5), (p value for all <0.05) (Table 5).

Table 4: ROC analysis of studied genes

Variables	AUC	p-value	95% confidence interval		Cutoff value	Sensitivity (%)	Specificity (%)	Accuracy (%)
			Lower bound	Upper bound				
IGF	0.755	0.001	0.622	0.888	32.3	71	70	70.5
FOXO	0.91	<0.001	0.829	0.990	0.99	81	82	81.5
LncRNA AF085935	0.966	<0.001	0.931	1.00	3.02	100	90	95
AFP	0.791	<0.001	0.674	0.908	26.9	71.1	98.6	84.85

IGF: Insulin-like growth factor, FOXO: Forkhead box class O, LncRNA: Long non-coding ribonucleic acid, AFP: Alpha-fetoprotein.

Table 5: Multivariate regression analysis for prediction of occurrence of HCC on HCV patients

Variables	p-value	Odds ratio	95% confidence interval for odds ratio	
			Lower bound	Upper bound
IGF	0.006	1.044	1.012	1.076
FOXO	<0.001	6.146	2.390	15.806
LncRNA	<0.001	18.824	8.196	43.234
AFP	0.004	4.08	1.074	15.5

IGF: Insulin-like growth factor, FOXO: Forkhead box class O, LncRNA: Long non-coding ribonucleic acid, AFP: Alpha-fetoprotein.

Discussion

Liver cirrhosis and HCC are major complication of HCV chronic infection [21]. HCC is one of the most common solid tumors, which rated as the third cancer-related mortality worldwide [22], [23]. Survival rate for HCC is very low due to lack of early diagnosis and reliable biomarkers, so new and accurate diagnostic biomarkers are needed. FOXO is an important transcriptional regulator that regulates multiple cellular functions as stress resistance, proliferation/apoptosis, and aging. Alteration in FOXO is involved in progression of several diseases and cancers [24].

Our results revealed significant upregulation in FOXO gene expression in HCC patients compared to the healthy control subjects. This agreed with the previous study which reported that FOXO promotes tumor cell invasion through the induction of matrix metalloproteinases [24]. Another study reported that FOXO acts as tumor suppressor genes that inhibit tumor formation under basal condition, on the other side, FOXO overactivation by multiple stimuli supports tumor growth and metastasis [25]. Moreover, another study reported that FOXO3a promotes gastric cancer invasion by regulating proteins involved in epithelial mesenchymal transition and extracellular matrix degradation. FOXO3a has prognostic value and represents a novel therapeutic target in inhibiting tumor metastasis [26].

IGF is a key modulator in the development and progression of cancers [27]. In our study, we also found upregulation of IGF level in HCV and HCC groups compared to control group and higher levels of IGF in HCC than in HCV patients. This coincides with the previous study that found higher IGF mRNA in HCV-infected patients as compared to the control [28]. Another study illustrated that IGF stimulates the growth and metastasis of HCC by inhibition of proteasome-mediated cathepsin B degradation [29]. Another study reported IGF-induced angiogenesis in many types of cancers. More over; IGF upregulates cyclin D1 expression in neural cell lines through the induction of JAK/STAT5 signaling pathway [30]. In addition, IGF trigger cell-cycle by inducing the expression of cyclins and cyclin-dependent kinases in nonneuronal cells Further study reported that HCV-related HCC activation of IGF signaling was significantly associated with mTOR signaling [31].

LncRNAs are the vital factors in the modulation of tumor development and progression of complex diseases. Some of the LncRNAs have been used as biomarkers in the screening of human cancers. In HCC, deregulated expressions of both protein-coding genes and LncRNAs have been recommended to have considerable potential in predicting the diagnosis and prognosis of HCC patients [16]. Hence, we demonstrated the association of LncRNA-AF085935 among HCC patients, HCV patients, and healthy controls. In our study, we found upregulation of LncRNA-AF085935 level in HCC patients compared to control group. The previous studies showed that LncRNAs are highly upregulated in HCC [32], and LncRNA-AF085935 expression can be used as prognostic biomarkers of HCC [33].

Conclusion

We can conclude that HCV virus can induce IGF which, in turn, can initiate carcinogenesis through upregulation of LncRNA-AF085935 as we found significant positive correlation between them in HCC patients, in addition, LncRNA-AF085935 is the most significant risk factor for occurrence of HCC. Since LncRNAs have a higher level on regulation of gene expression, LncRNA-AF085935 most probably can induce tumor progression through induction of FOXO gene. This is confirmed by our finding that LncRNA-AF085935 is significantly correlated with FOXO. FOXO1, IGF, and LncRNA-AF085935 were highly expressed in the serum of HCC patients, thus it can be considered as an useful candidate biomarkers for the screening of HCC.

Authors' Contributions

Participated in research design: Abeer Mostafa, Noha E. Ibrahim, and Dina Sabry. Sample

collection and clinical evaluations: Wael fathy and Amany Y. Elkazaz. Conducted experiments: Abeer Mostafa, Noha E. Ibrahim, and Dina Sabry. Analysis of data: Abeer Mostafa. Literature search: Noha E. Ibrahim and Amany Y. Elkazaz. Wrote or contributed to the writing of the manuscript: Noha E. Ibrahim and Abeer Mostafa. Revision of the article: Noha E. Ibrahim and Amany Y. Elkazaz. All authors read and approved the final manuscript.

Authors' Agreement/Declaration

All authors in agreement with the content of the manuscript. All authors declare that they have no known competing financial interests that could have appeared to influence the work reported in this paper.

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