

## Characterization of The Type of Response to Different HCV Antigens and Quantification of Viral Load in Newly Diagnosed Hepatocellular Carcinoma Patients

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### ABSTRACT

**Background:** Hepatitis C virus (HCV) is a blood born virus that is considered a major cause of chronic liver disease and hepatocellular carcinoma (HCC) worldwide. HCV is thought to induce HCC either indirectly or directly by the effect of its viral proteins on different host cell proteins and signaling pathways.

**Objective:** The aim of the study was to characterize the type of response to different HCV antigens, quantify HCV viral load, transforming growth factor- beta and miRNA 122 in patients with newly diagnosed Hepatocellular Carcinoma.

**Patients and methods:** This study was done on three groups: the first group consisted of 40 newly discovered hepatocellular carcinoma patients with HCV infection. The second group consisted of twenty HCV infected patients with other types of cancer (other than HCC). The third group consisted of 20 healthy individuals served as a control group. Serum was separated for detection of the four parameters. **Results:** TGF- $\beta$  showed a very weak negative correlation with the miRNA 122 serum levels that is statistically non-significant. Results also showed that miRNA 122 may not be useful in differentiating between liver cirrhosis from HCC patients and it is associated with the severity of the disease rather than the viremia count. **Conclusion:** Study showed no correlation between the four investigated parameters (HCV antigens, HCV viral load, TGF- $\beta$ - serum levels of miRNA 122) in an attempt for early diagnosis of HCV induced HCC.

**Keywords:** Hepatitis C - Hepatocellular Carcinoma- HCV antigens- Viral load- TGF-  $\beta$ - miRNA 122.

### INTRODUCTION

Hepatitis C virus (HCV) is a blood born virus which has a major contribution to chronic liver disease and hepatocellular carcinoma worldwide <sup>(1)</sup>. Hepatocellular carcinoma (HCC) is the fourth common cancer in Egypt and the second cause of death in both men and women. 71% of the Egyptian HCC cases were found to be HCV patients <sup>(2)</sup>.

HCV is thought to induce hepatocellular carcinoma either indirectly by causing chronic inflammation, insulin resistance, oxidative stress, fibrosis and genetic instability or directly by the effect of its viral proteins on various host cell proteins and signaling pathways <sup>(3)</sup>.

The core protein of HCV is found to cause hypermethylation of the promoter region of the tumor suppressor RBL2 gene leading to uncontrolled cell division <sup>(4)</sup>, it activates the Ras/Raf/MAP Kinase signal transduction pathway through the activation of Raf-1 kinase, it also activates  $\beta$ - catenin / Wnt signaling pathway through the activation of the  $\beta$ - catenin and its stabilization <sup>(5)</sup>. The core protein also is found to inhibit the growth inhibitory pathway of the cytokine TGF- $\beta$  and shifting its activity to the pro-fibrinogenic property of the TGF- $\beta$  by interacting with SMAD3 directly <sup>(6)</sup>. Moreover, HCV core protein inhibits the expression of the PRAR- $\alpha$  receptors in the liver leading to hepatic steatosis <sup>(7)</sup>. The core protein also inhibits the activation of the anti-apoptotic NF- $\kappa$ B pathway leading to the induction of apoptosis in hepatocytes <sup>(8)</sup>. The HCV core protein interacts with the endoplasmic reticulum and the

mitochondrial membrane; interfering with the electron transport chain causing oxidative stress <sup>(9)</sup>.

E1 and E2 glycoproteins of the HCV are found to trigger epithelial -mesenchymal trans-differentiation induction through enhancing the signaling pathways of TGF- $\beta$  and VEGF <sup>(10)</sup>. E1/E2 proteins are found to interfere with the electron transport chain (ETC) in the mitochondria, decrease the content of NADPH and increase the production of reactive oxygen species from ETC complex I substrates leading to reduced activity of ETC complex I <sup>(11)</sup>. E2 interferes with the host immune response by inhibiting protein kinase R and inhibiting the activation of natural killer cells and T cells <sup>(12)</sup>. E2 protein of HCV has been found to enhance cell proliferation through activation of the MAPK/ERK signaling pathway <sup>(13)</sup>.

The non-structural protein NS2 of HCV induces cyclin E expression and activates cyclin D/ CDK4 which is a significant cell cycle checkpoint for the transition from the G1 phase to S phase promoting proliferation. It can also prevent apoptosis by interfering with the p53 pathway where it phosphorylates Chk2 but doesn't phosphorylate the p53, thus the p53 is retained in the cytoplasm <sup>(14)</sup>.

The non-structural protein NS3 of HCV is found to activate the Ras/Raf/MAPK signaling pathway by inhibiting protein kinase A which has negative feedback mechanism on Ras/Raf/MAPK pathway. NS3 prevents the translocation of protein kinase A from cytoplasm to

nucleus<sup>(15)</sup>. NS3 together with its cofactor NS4a interacts with ATM, Check point kinase preventing the activation of tumor suppressor gene p53, thus prevent the activation of its downstream target tumor suppressor protein 21 and prevent the expression genes responsible for DNA repair<sup>(16)</sup>. NS3 is found to reduce the expression of tumor suppressor PPM1A by interacting and locating it in the cytoplasm promoting its ubiquitination and proteosomal degradation<sup>(17)</sup>.

The non-structural protein NS5A of HCV is found to activate the NS5A is found to activate wnt/ $\beta$ -catenin signaling pathway increasing hepatocytes proliferation. NS5A inhibits apoptosis by enhancing the PI3K/ Akt survival regulatory pathway<sup>(18)</sup>. NS5A interacts directly and sequesters p53 preventing its translocation from the cytoplasm to the nucleus leading to the inhibition of p53 induced apoptosis, it also interacts with pro-apoptotic Bax and inhibits apoptosis<sup>(19)</sup>. Also, NS5A is found to inhibit the TGF- $\beta$  signaling pathway, targeting its anti-proliferative and pro-apoptotic function. It directly interacts with TGF- $\beta$  receptor 1 (TGFBR1), inhibiting its phosphorylation and prevents the translocation of smad2 to the nucleus leading the inhibition of the growth inhibiting pathway of TGF- $\beta$ <sup>(20)</sup>. NS5A overexpression triggers the release of ROS; it induces the release of calcium from the ER<sup>(21)</sup>. Moreover, NS5A reduces the expression of mitotic spindle protein ASPM through interfering with the protein kinase R (PKR)-p38 pathway, thus causing aberrant mitosis and chromosomal instability<sup>(22)</sup>. The non-structural protein NS5B of HCV interacts with retinoblastoma, promoting its degradation resulting in the dissociation from its bound E2F transcription factor and hence E2F activation leading to cell cycle progression and proliferation<sup>(23)</sup>.

The aim of the current study was to characterize the type of response to different HCV antigens, quantify HCV viral load, transforming growth factor- beta in patients with newly diagnosed Hepatocellular Carcinoma.

## MATERIAL AND METHODS

This case control study included a total of 80 subjects, 60 of them were HCV infected patients with a cancer and 20 normal controls, attending at the Outpatient Clinic at National Cancer Institute. This study was conducted in May 2015.

### Ethical approval:

**Approval of the ethical committee of Faculty of Pharmacy, Cairo University was obtained. Written informed consent from all the subjects were obtained.**

The included subjects were divided into three groups; **Group 1** consisted of 40 newly discovered hepatocellular carcinoma patients with HCV infection (26 males and 14 females) with an age range from 55-70 years old, **Group 2** consisted of twenty HCV infected patients

with other types of cancer (not hepatocellular carcinoma) (10 males and 10 females) with an age range 40-55 years, and **Group 3** consisted of 20 healthy individuals served as a control group (9 males and 11 females). For all the study groups, full medical history was taken, and blood sampling was done.

Antibodies for different particles of HCV were detected by Multisure HCV antibody rapid qualitative test kit, MP Diagnostics according to the manufacturer's instructions.

Transforming growth factor-Beta (TGF- $\beta$ ) was measured for HCC patients using TGF- $\beta$  ELISA kit, BioSource Lab according to the manufacturer's instructions.

The viral load of HCV for group (1) HCV induced HCC and group (2) HCV with other cancers were detected by real time PCR technique. The kit used in the RNA isolation was DNA/ RNA virus Mini Kit (STRATEC Molecular GmbH, Berlin, Germany) according to the manufacturer's instructions. The HCV PCR Fluorescence Quantitative Detection Assay Kit is then used for reverse transcription of viral RNA and subsequent PCR amplification of the target cDNA and its fluorescent detection using specific Taqman- MGB probes according to the manufacturer's instructions.

The Serum levels of miRNA 122 in the three groups were also measured by real time PCR. The GeneJet RNA purification kit, ThermoFisher Scientific was firstly used for RNA purification according to the manufacturer's instructions. The cDNA was then synthesized using Taqman MicroRNA Reverse Transcription kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. Primers used forward primer 5'GCTCGACCTCTCTATGGGC and miRNA 122 reverse primer: 5'TTAAGCCCTGCGTGTCTCC. The microRNA 122 was finally amplified and detected using Taqman Universal Master Mix II (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions.

### Statistical analysis

SPSS version 23.0 was used for data management and data analysis. Mean  $\pm$  standard deviation with median and range when appropriate described quantitative data. Numbers with percentages described qualitative data calculated by Chi-square test. The Independent T-test and one-way ANOVA test were performed for the numerical variables displaying normal distribution. The Mann-Whitney U-test and Kruskal Wallis Test were performed for the numerical variables not displaying normal distribution. If continuous variables are in parametric data correlation calculated by Pearson correlation, but non-parametric data correlation calculated by Spearman correlation. The P-value will be set at 0.05 and all of the comparisons will be two-tailed.

**RESULTS**

**Table (1):** Laboratory results of viral load, TGF-β and miRNA 122 variables among the three groups of the study

	Viral Load	TGF-β	miRNA 122
<b>Group (1)*</b> 40 HCC patients	157± 490	87.5 ± 75.62	23.5± 52.5
<b>Group (2)*</b> 20 other cancers	32.3 ± 31.8	-----	57.5 ± 164.8
<b>Group (3)</b> 20 healthy controls	-----	-----	0.00

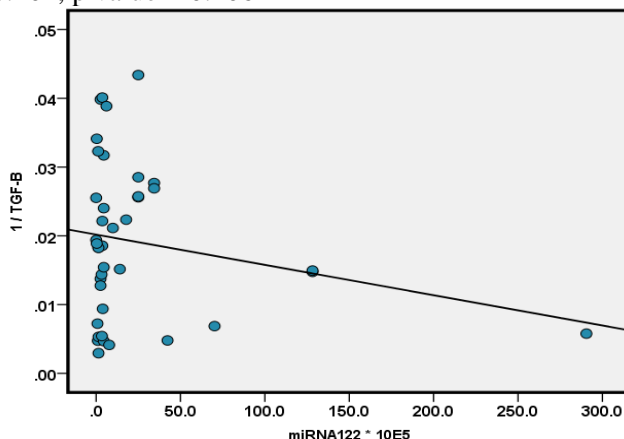
\*values are in mean± SD

**Table (2):** Correlations between parameters in the first group (HCC group)

		Log value of PCR viral load	miRNA122 * 10 <sup>5</sup>
Log value of PCR viral load	r value*		-0.064
	p value		0.726
	N		32
miRNA122 * 10 <sup>5</sup>	r value	-0.064	
	p value	0.726	
	N	32	
1 / TGF-B	r value	-0.054	<b>-0.204*</b>
	p value	0.769	<b>0.206</b>
	N	32	<b>40</b>

\* The size of r indicates the strength of the correlation.  
 \*-0.5 < r < 0 indicates a very weak negative correlation.  
 \*p-value ≤ 0.05 is significant.

There was a very weak negative correlation between miRNA122 level and 1/TGF-B, but it was not significant, r = -0.204, p value = 0.206



**Figure (1):** Scatter plot showing correlation between 1 / TGF-B and miRNA122 \* 10<sup>5</sup> in group (1) HCC patients

**Table (3):** Comparison of Log value of PCR viral load according to HCV antibody assay in group (1) HCC patients.

	N	mean± SD	Median	p-value
<b>Cp, NS3, NS4</b>	24	3.5± 1.35	3.53	0.705*
<b>Cp, NS3, NS4, NS5</b>	6	3.6± 0.80	3.57	

\*p-value ≤ 0.05 is significant

**Table (4):** Comparison of 1 / TGF-B according to HCV antibody assay in group (1) HCC patients.

	N	mean± SD	Median	p-value
<b>Cp, NS3, NS4</b>	28	0.018 ± 0.011	0.0183	0.683*
<b>Cp, NS3, NS4, NS5</b>	10	0.020 ± 0.013	0.0188	

\*p-value ≤ 0.05 is significant

**Table (5):** Comparison of miRNA122\*10<sup>5</sup> according to HCV antibody assay in group (1) HCC patients.

	N	mean± SD	Median	p-value
<b>Cp, NS3, NS4</b>	28	24.57 ± 58.8	3.54	0.351*
<b>Cp, NS3, NS4, NS5</b>	10	24.44 ± 38.71	10.90	

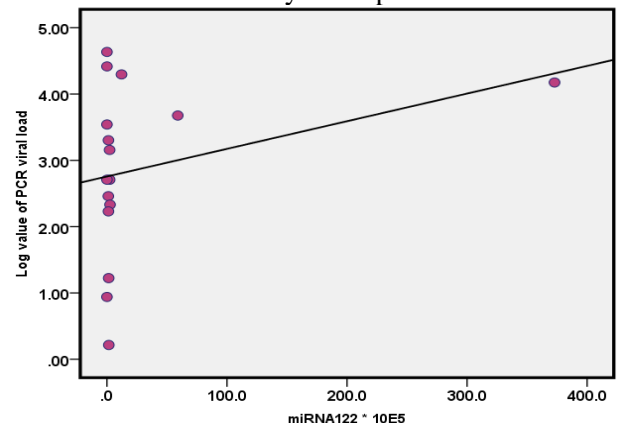
\*p-value ≤ 0.05 is significant

**Table (6):** Correlations between miRNA122 and PCR viral load in group (2) other cancers patients.

miRNA 122*10 <sup>5</sup>		Log value of PCR viral load
	r value	0.300*
	p-value	0.259*
	N	16

\*p-value ≤ 0.05 is significant.

\* 0 < r < 0.5 indicates a very weak positive correlation.



**Figure (2):** Scatter plot showing correlation between Log value of PCR viral load and miRNA122 \* 10<sup>5</sup>

**Table (7): Comparison of miRNA 122 \*10<sup>5</sup> among group 1 (HCC patients) and group 2 (other cancers patients)**

	N	Median	p-value
Group (1) HCC patients	40	4.216	0.063*
Group (2) other cancers patients	20	1.471	

\*p-value ≤ 0.05 is significant

Median value of miRNA122 was tempting to be higher in HCC group as compared to Other cancers (HCV) group, but p value was not significant = 0.063

## DISCUSSION

Among the Egyptian people, the incidence of hepatocellular carcinoma is increasing. In Egypt, 70% of the HCC patients are chronic HCV induced. 80% of the HCC patient had pre-existing cirrhosis (end stage liver disease). The late diagnosis of the disease either due to the lack of symptoms or the absence of monitoring for high risk patients account for the high mortality rate among HCC patients. Early diagnosis of HCC can therefore have a great impact on the responsiveness to the treatment and hence a reduction in the mortality rate.

The current study was conducted with the aim of characterizing the type of response to different HCV antigens, quantification of HCV viral load, quantification of transforming growth factor- beta (TGF-β), and quantification of the serum level of miRNA 122 in early diagnosed hepatocellular carcinoma patients in an attempt to find whether there is a correlation between these four different variables for an early diagnosis of HCC.

In our study, the majority of HCC patients (76.9%) were stage B (intermediate) based on BCLC scoring system.

The current study detected antibodies specific to HCV core, NS3, NS4 and NS5 antigens. The results showed 70% of patients having antibodies to core, NS3 and NS4, 25% of patients having antibodies to core, NS3, NS4 and NS5, 2.5% of patients having antibodies to core and NS3 and 2.5% of patients having antibodies to core only.

According to the study done by Wang<sup>(24)</sup>, it showed that presence of different HCV proteins is found to have different significance. The detection of Core Protein indicates the exposure of HCV showing possible carcinogenic effects. While detecting NS3, NS4, NS5 indicates the early exposure and active multiplication of HCV in the host cells. Absence of any of these non-structural proteins shows recovery from infection with high rates of mutation.

In correlation to Wang<sup>(24)</sup>, our study thus showed 100% of the patients have carcinogenic tendency, 70% of

the patients have core and NS3 and NS4, missing NS5 and its replicase activity indicating recovery from infection with high rates of mutation and carcinogenic tendency.

The current study also measured TGF-β as an indicator of fibrosis in HCC patients with hepatitis C infection. Our results found a very weak negative correlation with the miRNA 122 serum levels (r= -0.204) that is statistically non-significant (p=0.206). The negative poor correlation is supported by the study done Trebicka *et al.*<sup>(25)</sup> by that showed that as the progression of fibrosis occurs; less miRNA 122 is released into the circulation due to the loss of the hepatic cells in end stage liver disease and the decrease in the hepatic miRNA 122 level indicating that miRNA 122 is not a proper marker for the progression of fibrosis. Our results may be statistically non-significant due to the small sample size and the variability of the miRNA 122 between the patients.

On the other side, the study done by Kong *et al.*<sup>(26)</sup> suggested that TGF-β could represent fibrogenesis rather than fibrosis. The study showed a negative correlation between the serum TGF-β and the fibrotic score, where cirrhotic patients showed the least serum TGF-β values. Kong and his colleges concluded that at a certain point an active TGF-β may not correlate with the fibrosis score. The fibrogenesis is a long process involving the activation of hepatostellate cells by various cytokines and their transformation into myofibroblast and collagen formation while fibrosis is the sum up of this lengthy process that took place previously.

The present study measured the serum miRNA 122 levels in the three groups of HCV induced HCC (group 1), HCV infected patients with other different types of cancers (group 2) and normal control individuals (group 3). The results showed a significant difference in the serum levels of miRNA 122 between group 1 and 3 (p< 0.001) and between group 2 and 3 (p< 0.001) suggesting a significant rise in the serum level of miRNA among both patients' groups compared to the normal individuals.

Our results were supported by the findings of other studies; Wang *et al.*<sup>(27)</sup> showed an increase in the serum levels of miRNA 122 as compared to healthy controls. The results of Gramantieri *et al.*<sup>(28)</sup> also found a downregulation in the expression of miRNA 122 in the HCC tissues. Concluding that miRNA 122 is liver specific miRNA that is normally abundant in the hepatocytes. As the hepatic cells are injured, the miRNA 122 is released into the circulation causing a rise in its serum level.

Moreover, a study done by Qi *et al.*<sup>(29)</sup> evaluated the serum levels of miRNA 122 in the HCC patients before and after surgical removal of tumors. They found that miRNA 122 serum levels were significantly reduced after surgery suggesting that the circulating levels of miRNA 122 were originated from the HCC tissue.

On the other hand, three patients with ascites in our study showed a significant decrease in serum miRNA 122

levels which is consistent with the results of the study conducted by **Waidmann et al.**<sup>(30)</sup> which found that patients with complications of ascites, spontaneous bacterial peritonitis and hepatic-renal syndrome showed a significant reduction in the serum levels of miRNA 122 than those without these complications. **Waidmann et al.**<sup>(30)</sup> also revealed poor prognosis and higher mortality rates in patients with progressive liver disease showing lower serum levels of miRNA 122.

In addition, **Köberele et al.**<sup>(31)</sup> studied the plasma levels of miRNA 122 expression in end stage liver disease, and found that miRNA 122 plasma levels can indicate the remaining functional liver tissue in end-stage liver disease. While **Waidmann et al.**<sup>(30)</sup> showed that serum miRNA 122 is used as indicator of hepatic cell injury in earlier stages of liver diseases.

Our results also showed an increase in median value serum levels of miRNA 122 patients group 1 than in group 2, statistically non-significant ( $p=0.063$ ). These results are consistent with the data obtained by the study done by **Köberele et al.**<sup>(31)</sup> that found non-significant rise in the mean serum levels of miRNA 122 in HCC patients compared to liver cirrhosis without HCC, concluding that miRNA 122 may not be useful in differentiating between liver cirrhosis from HCC patients.

On the contrary, other studies showed a significant rise in the mean serum miRNA 122 levels in HCC patients as compared with cirrhotic patient group<sup>(32)</sup>

The current study also measured the viral load in all of the HCV infected patients; our results showed no correlation between the HCV viral load in HCC patients and the serum levels of miRNA 122. Our results are consistent with the results obtained by the study done by **Correia et al.**<sup>(33)</sup>. Our results concluded that the miRNA 122 is associated with the disease severity rather than the viremia count.

On the other hand, our results showed a positive fair association between serum miRNA 122 level and the HCV viral load ( $r=0.300$ ) in HCV infected patients having other types of cancers which is non-statistically significant ( $p=0.259$ ).

The positive correlation is supported by the study **Masaki et al.**<sup>(34)</sup> that showed the 5'UTR of the HCV genome recruit several cellular factors that are important for viral translation and/or RNA replication including eukaryotic initiation factor 3 (eIF3), poly(rC)- binding protein 2 (PCBP2), and microRNA 122. The study showed that miRNA 122 displaces PCBP2, thus reducing the HCV genomes involved in translation, while increasing the availability for RNA synthesis.

Our results may be statistically non-significant due to the small sample size used in the study.

In attempt to make a comparison between different HCV antigens and HCV viral load, TGF- $\beta$  and miRNA 122, our results showed no relation between these

different variables ( $p=0.705$ ), ( $p=0.683$ ) and ( $p=0.351$ ) respectively.

## CONCLUSION

Based on the results of our study, all the HCV patients in the study showed the presence of antibodies for the core protein of HCV that plays an important role in carcinogenesis, while 70% of the patients have antibodies for core and NS3 and NS4, missing NS5 and its replicase activity showing recovery from infection with high rates of mutation and carcinogenic tendency.

Our results showed miRNA 122 may not be useful in differentiating between liver cirrhosis from HCC patients and it is associated with the severity of the disease rather than the viremia count.

Moreover, our results showed that TGF- $\beta$  has a very weak negative correlation with the miRNA 122 serum levels that is statistically non-significant.

We found in conclusion that there is no correlation between the four parameters under investigation (HCV antigens, HCV viral load, TGF- $\beta$ - serum levels of miRNA 122).

## RECOMMENDATIONS

Our study needs further verification in a larger sample size. Liver biopsy may be also performed to confirm the stage of liver fibrosis. Moreover, the addition of other biomarkers that could be more specific for HCC and could be used as for comparison between groups.

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