Correlation of NF-kB Serum Level with the Expression Pattern of Microrna-221 in a Sample of Hcv-Exposed Iraqi Patients

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Abstract

Background: Hepatitis C virus (HCV) has the ability to change cellular messenger RNA transcription and translation by stimulating the synthesis of cellular microRNAs (miRNAs) that impair immune response and facilitate viral reproduction. One of the most important members of the immune response against HCV is nuclear factor-kappa B (NF-κB), which is regulated by cellular miRNAs. **Aims:** we aimed to investigate the correlation of NF-κB serum level with circulatory miRNA-221 (miR-221) fold change in HCV-exposed individuals. **Materials and Methods:** Serum level of NF-κB in 88 samples (22 patients with persistent HCV infection, 22 individuals with spontaneous HCV virus clearance, 22 individuals treated with direct-acting antivirals (DAAs) drugs, and 22 uninfected apparently healthy blood donors as control) was measured by enzyme-linked immunosorbent assay. Reverse transcriptase–polymerase chain reaction was used to quantify the expression fold of circulatory miR-221. **Results:** The results showed that there was a significant decrease in the mean level of NF-κB at $P < 0.000$ among HCV-exposed patients (2.0058 ng/ml) as compared to the control group (2.9841 ng/ml). The mean fold change of miR-221 was significantly upregulated about six more times among HCV-exposed patients (mean = 6.3545) compared to the control group (mean = 1.1864). Furthermore, the mean ± standard deviation of miR-221 fold change in patients with persistent HCV infection was significantly higher compared to patients cured after DAA therapy ($P = 0.048$), there was a weak negative correlation ($r = -0.246$, $P = 0.021$) between NF-κB serum level and miR-221 folding level. **Conclusion:** HCV infection disrupts NF-κB activation, resulting in dysregulation of miR-221 that persists long after the virus has been cleared. Thus, quantification of serum NF-κB and miR-221in HCV-exposed patients could be used as noninvasive prognostic marker during long-term follow-up. Furthermore, a miRNAs profile analysis can help distinguish HCV-exposed from healthy individuals.

Keywords: Hepatitis C virus, microRNA-221, nuclear factor-kappa B

Introduction

Hepatitis C virus (HCV) is an RNA virus belonging to the family Flaviviridae. It has been considered a worldwide public health concern and a significant risk factor for the development of severe progressive liver disease due to its ability to induce a persistent inflammatory response.[1] Although its a high propensity for chronicity, about one-third of patients with an acute HCV infection will clear the virus spontaneously by mounting a humoral and cellular immune response.[2] Moreover, the development of interferon-free direct-acting antivirals represents a significant step forward in the treatment of HCV infection. Still, minimal information is available regarding the onus of the immune system after HCV clearance.

Accumulating evidence suggests that HCV infects not only hepatocytes but also other extrahepatic reservoirs as B-cells, T-cells, and macrophages/monocytes.[3] However, little is known about the impact of HCV on infected immune cells and how the virus hijacks cellular signaling pathways and transcription factors (TFs) to their own advantage.

Nuclear factor-kappa B, a short synonym for nuclear factor κ-light-chain enhancer of activated B-cells (NF-κB), pathway, in particular, appears to be a tempting target for common human viral pathogens, since, it constitutes a family

Access this article online

Quick Response Code:

Website: http://www.mmjonweb.org

DOI: 10.4103/mj.mj_20_22

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Submitted: 01-Jul-2022 **Revised:** 24-Jul-2022 **Accepted:** 29-Jul-2022 **Published:** 07-Aug-2023

How to cite this article: Al-Salihy SR, Al-Shawk R, Al-Waysi SA, Rasheed MN. Correlation of NF-kB serum level with the expression pattern of microrna-221 in a sample of hcv-exposed Iraqi Patients. Mustansiriya Med J 2023;22:20-5.

of TFs that influence the expression of hundreds of genes primarily involved in many physiological processes, such as cell proliferation, cell survival, inflammation, and immune response.[4]

NF-κB consists of five subunits including NF-κB1 (p50), NF-κB2 (p52), Rel A (p65), Rel B, and c-Rel. NF-κB subunits, which can form different types of homodimers and heterodimers, remain sequestered in the cytoplasm of unstimulated cells by binding to the inhibitor of κB proteins (IκB).[5] The NF-κB signaling pathway can be activated by a variety of stimuli, including recognition of pathogen-associated molecular patterns by host-derived pattern recognition receptor and proinflammatory cytokines, ultimately lead to phosphorylation and degradation of IκB, releasing NF-κB dimers to be translocated into the nucleus.[6]

MicroRNAs (miRNAs), a family of gene expression regulators, are short noncoding RNAs (average of 18–22 nucleotide), that regulate gene expression posttranscriptionally through sequence-specific interactions with the 3`untranslated regions of their target messenger RNAs (mRNAs). They silence genes primarily by degrading target mRNAs or inhibiting translation.[7,8]

Recently, it has been found that viruses can change cellular mRNA transcription and translation by stimulating the synthesis of cellular miRNAs that impair innate defense pathways, so facilitating viral reproduction.[9] One such virus, HCV, dysregulates many miRNAs that influence its life cycle and immune evasion.[10,11] Many reports have linked the upregulation of miRNA-221 (miR-221) with HCV-induced liver fibrosis and hepatocellular carcinoma.[12-14] However, it is still unclear how miRNAs play a role in HCV pathogenesis.

Furthermore, new research suggests that miRNAs have a role in the regulation of the NF-κB signaling pathway through different mechanisms.[15,16]

Given the fact that the functional link between the NF-κB signaling pathway and miRNAs is critical for immune response control, study aimed to investigate the correlation between circulatory miR-221 level and NF-κB serum level.

Materials and Methods

Study subjects

The present case–control study was conducted in Hepatology and Gastroenterology Teaching Hospital in the medical city/Baghdad/Iraq, during the period extended between December 1, 2020, and August 30, 2021. A total of 88 individuals participated in this study. Of them, 66 patients were exposed to HCV (24 males and 42 females) with an age range of 20–68 years old. HCV-exposed patients were subcategorized into three groups each with 22 participants; Group 1 represents chronic HCV infection patients, Group 2 includes individuals who had spontaneously recovered HCV infection (based on positive anti-HCV antibodies and negative HCV polymerase chain reaction [PCR]), and Group 3 comprises HCV-patients who showed sustained antiviral response (based on aviremia 12 weeks after completion of HCV antiviral therapy). The remaining 22 participants were healthy blood donors as a control. They were considered to be matched with HCV-exposed patients in terms of age and gender.

Excluding criteria included patients with no other causes of liver disease, autoimmune or metabolic disorder, coinfection with hepatitis B virus and/or human immunodeficiency virus, liver steatosis, malignancies, and current alcohol abuse.

Sample collection

Approximately 5 ml of whole blood was drawn by venipuncture from each participant and left for serum separation. Sera were separated by centrifugation for 10 min at 3000 rounds/min (rpm). Then, 600 µl of serum was aspirated and transferred to Eppendorf plastic tubes containing 400 µl of Trizol for RNA extraction and stored at −20°C till use. The remaining sera were divided into aliquots of (250 µl) and used for immunological tests.

Viral testing

According to the manufacturer's instructions, qualitatively detecting anti-HCV antibodies was done using a fourth-generation enzyme-linked immunosorbent assay (ELISA) kit (bio ELISA HCV fourth-generation Fortress diagnostics, UK). HCV RNA analysis was measured by quantitative PCR assay for those who gave a positive result.

Detection of human nuclear factor-kappa B level in serum

The concentration of human NF-κB in sample was determined using sandwich-ELISA kit (Human NF-κB, Sunlong, China) according to the manufacturer's instructions.

Gene expression analysis of microRNA-221 by quantitative polymerase chain reaction

The total RNA was extracted from samples according to the protocol of TRIzol™ Reagent (Thermo Scientific, USA). Complementary DNAs for miR-221 and RNU43 (reference gene) were produced using GoScript™ Reverse Transcription System (Promega, USA) as per manufacturer's protocol. Quantus™ Fluorometer was used to determine the quality and quantity of produced cDNA for downstream application. Amplification of miR-221 and RNU43 was done by two-step reverse transcriptase–PCR as shown in Table 1. Relative quantification of miR-221 was calculated using $2^{-\Delta\Delta CT}$ formula. Primers are listed in Table 2.

Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-27 (Statistical Packages for the Social Sciences-version 27). Data were presented in simple measures of frequency, percentage, mean, standard deviation (SD), and range (minimum–maximum values).

The significance of difference of different means (quantitative data) was tested using Student's *t*-test for difference between two independent means or ANOVA test for difference among more than two independent means. The significance of difference of different percentages (qualitative data) was tested using Pearson's Chi-square test with the application of

Yate's correction or Fisher's exact test whenever applicable. Statistical significance was considered whenever the $P \leq 0.05$. Phenotypic Odds ratio calculated by the aid of medcalc.net. Pearson correlation was calculated for the correlation between two quantitative variables with its *t*-test for testing the significance of correlation.

Results

The gender and age groups of the study groups are listed in Table 3. Generally, the rate of females was higher than males in each of G1, G2, and G3 (63.7% vs. 36.3%), as well as among controls (59.1% vs. 40.9%). However, the difference among the study groups was failed to reach the level of statistical significance ($P = 0.986$). Regarding the age, the mean age ± SD was (38.77 ± 13.26) years in G1, (39.18 ± 13.22) years in G2, (45.64 ± 14.23) years in G3, and (41.45 ± 11.05) years in the control group. Therefore, the distribution of the study group according to their age showed a predominance in HCV infection among patients older than 40 years. Nevertheless, the difference in the age among study groups was statistically insignificant ($P = 0.336$).

Table 1: Steps and conditions of microRNA-221 by reverse transcription-polymerase chain reaction amplification

Step	Temperature (°C)	Time (m: s)	Cycle
First step			
Denaturation	70	5	1
Hold	4	10	
Cool and spin			
Annealing	25	05:00	1
Extension	42	60:00	
Enzyme inactivation	70	15:00	
Hold	4	10	
Second step			
Initial denaturation	95	05:00	1
Extension	95	00:15	50
Annealing	55	00:15 acquiring on green	
Extension	72	00:15	

Serum level of nuclear factor-kappa B among hepatitis C virus-exposed patients and control groups

Intriguingly, the present results revealed a significant decrease in the mean level of NF-κB at $P < 0.000$ among HCV-exposed patients (2.0058 ng/ml) as compared to the control group (2.9841 ng/ml). Moreover, the differences in the means ± SD level of NF-κB between (G1, G2, and G3 vs. control) groups were significant with $P = 0.0001$, 0.033, and 0.003, respectively. While the differences in means ± SD level either between (G1 vs. G2), (G1 vs. G3), or (G2 vs. G3) were statistically insignificant. Details are shown in Tables 4 and 5.

Expression profile of MicroRNA-221

To investigate the level of miR-221expression, quantitative real-time PCR was used to measure the concentration of circulating miR-221 in sera of participants. The molecular investigation was performed to detect the amplification plots of the target gene (miR-221) and reference

Table 2: Primer sequence for microRNA gene expression

Primer name	Primer sequence
miR-221-3p-RT	5`- GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGC ACCAGAGCCAACGAAACC-3`
miR-221-3p-F1	5`- GGAGCTACATTGTCTGCTG-3`
RNU43_RT	5`- GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGC ACCAGAGCCAACAATCAG-3`
RNU43_F	5`- GTGAACTTATTGACGGGCG-3`
Universal reverse	5`- GTGCAGGGTCCGAGGT-3`

miR: MicroRNA

Table 3: Distribution of the study groups according to age and gender

Variables	HCV-exposed subject			Control, *n* (%)	*P*
	G1, *n* (%)	G2, *n* (%)	G3, *n* (%)		
Age (years)					
20-39	9 (40.9)	9 (40.9)	11 (50)	11 (50)	0.336^
40-68	13 (59.1)	13 (59.1)	11 (50)	11 (50)	
Range	20-68	20-60	20-68	23-60	
Mean±SD	38.77±13.26	39.18±13.22	45.64±14.23	41.45±11.05	
Gender					
Male	8 (36.3)	8 (36.3)	8 (36.3)	9 (40.9)	0.986*
Female	14 (63.7)	14 (63.7)	14 (63.7)	13 (59.1)	

*Insignificant difference between proportions using Pearson's Chi-square test at 0.05, ^Insignificant difference among more than two independent means using ANOVA-test at 0.05. G1: Chronic HCV infection, G2: Spontaneously recovered HCV, G3: Patients with SVR response. SD: Standard deviation, HCV: Hepatitis C virus, SVR: Sustained virologic response

gene (RNU43) to find the threshold cycle (Ct) value that are inversely associated with the amount of starting template and calculate melting temperature curve as in Figures 1 and 2.

Investigating the fold change in the expression level of miR-221 in sera of studied groups showed that the miR-221 level was significantly upregulated about six more times among HCV-exposed patients (mean = 6.3545) compared to control (mean = 1.1864), Table 6.

In detail, we noticed a significant upregulation of miR-221 level in each of G1, G2, and G3 compared to healthy control ($P = 0.0001$), ($P = 0.001$), and ($P = 0.001$), respectively. Furthermore, miR-221fold change in G1 was significantly higher compared to G3 ($P = 0.048$), but insignificantly different compared to G2 ($P = 0.22$). Similarly, the miR-221 level was also insignificantly upregulated among G2 compared to G3 ($P = 0.346$). Data are shown in Table 7.

Table 4: Serum level of nuclear factor κ-light-chain enhancer of activated B cells (ng/mL) between hepatitis C virus-exposed patients and control groups

Study group	NF-κB serum concentration			*t*-test	*P*
	n	Mean±SD	SEM		
HCV-exposed patients	66	2.0058±1.00180	0.12331	−3.801	0.000#
Control	22	2.9841±1.16964			

#Significant difference between two independent means using student's *t*-test at 0.05 level. HCV: Hepatitis C virus, SD: Standard deviation, SEM: Standard error of the mean, NF-κB: Nuclear factor κ-light-chain enhancer of activated B cells

Table 5: Distribution of serum levels of nuclear factor κ-light-chain enhancer of activated B cells (ng/mL) among study groups

Study group	Concentration of NF-κB (ng/mL), mean±SD (range)	*P* value compared to		
		Control	G3	G2
G1	1.917±0.613 (0.993-3.281)	0.0001#	0.931	0.433
G2	2.161±1.307 (1.069-6.471)	0.033#	0.529	-
G3	1.939±0.992 (0.706-4.998)	0.003#	-	-
Control	2.984±1.170 (1.740-5.759)	-	-	-

#Significant difference between two independent means using Student's *t*-test at 0.05 level. G1: Chronic HCV infection, G2: Spontaneously clarified HCV, G3: Patients with SVR response. SD: Standard deviation, HCV: Hepatitis C virus, SVR: Sustained virologic response, NF-κB: Nuclear factor κ-light-chain enhancer of activated B cells

Table 6: Serum level of microRNA-221 between hepatitis C virus-exposed patients and control groups

Study group	Serum fold change of miR-221		*t*-test	*P*
	n	Mean±SD		
HCV-exposed patients	66	6.3545±5.31486	7.444	0.000#
Control	22	1.1864±1.08991		

#Significant difference between two independent means using Student's *t*-test at 0.05 level. SD: Standard deviation, HCV: Hepatitis C virus, miR: MicroRNA

Correlation between microRNA-221 fold change and nuclear factor-kappa B serum level

The results obtained during the present study suggested that there was a link between NF-κB serum level and miR-221 folding level. Intriguingly, NF-kB serum level was significantly decreased among HCV-exposed individuals in conjunction with significant increase in circulating miR-221 level. In addition, our results showed that there was an inverse correlation ($r = -0.246$, $P = 0.021$) between NF-κB serum level and miR-221folding level as shown in Figure 3.

Discussion

Innate immune responses provide the first line of defense against viruses. NF-κB has been dubbed as "key modulator of the immune response" due to its pivotal role in immunological and pro-inflammatory activity, as well as to its role as a linkage between innate and adaptive immunity.[6]

In the present study, the NF-κB serum level was significantly decreased among HCV-exposed patients compared to healthy individuals. Moreover, the NF-κB serum level was lower in the chronic HCV-patients group than in others; however, the differences were statistically insignificant.

It is well known that NF-κB is an intracellular factor and its activation had previously been linked to apoptosis, being antiapoptotic or proapoptotic depending on the kind of cell, in which it is produced and the stimulus that prompts its activation.[17,18]

Song *et al*.[18] demonstrated that "HCV core protein could trigger the activation of NF-κB pathway in macrophages which in turn may contribute to the chronically activated, persistence state of HCV-infected cells." Not unexpectedly, the prolonged activation of the NF-κB pathway maintained by HCV contributes to oncogenic transformation, thus its presence in serum might reflect the amount of cell death.

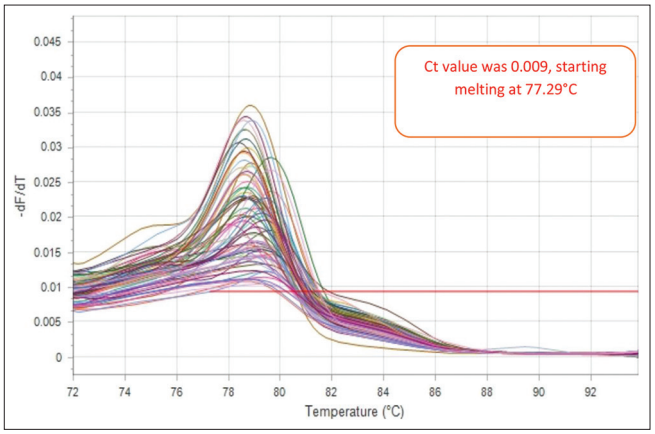

Figure 1: The miR-221 expression melting curve from 72°C to 95°C. miR-221: miRNA-221

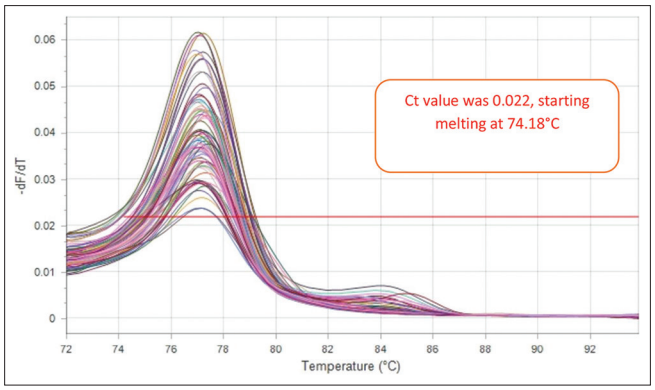

Figure 2: The RNU expression melting curve from 72°C to 95°C

Table 7: Serum fold change expression level of microRNA-221 among studied groups

Study groups	miR-221 folding, mean±SD (range)	*P* value compared to		
		Control	G3	G2
G1	8.159±6.196 (1.20-19.90)	0.0001#	0.048#	0.220
G2	6.118±4.561 (1.80-17.30)	0.0001#	0.346	-
G3	4.786±4.711 (0.30-18.60)	0.001#	-	-
Control	1.186±1.090 (0.10-5.10)	-	-	-

#Significant difference between two independent means using Student's *t*-test at 0.05 level. G1: Chronic HCV infection, G2: Spontaneously recovered HCV, G3: Patients with SVR response. SD: Standard deviation, HCV: Hepatitis C virus, SVR: Sustained virologic response, miR: MicroRNA

The results of this investigation contraindicate the results obtained by Neuman and Cohen.[17] They found that the serum level of tumor necrosis factor-α, interferon-γ, and of NF-κB were elevated among HCV patients compared to control. This contraindication may be due to the difference in virus genotypes or genetic variation of participants.

Investigating the expression level of miR-221 in sera of studied groups showed that the miR-221 level was significantly upregulated about six more times among HCV-exposed patients with respect to healthy control. In more detail, the chronic HCV patients showed significantly higher levels than those who showed sustained virologic response after treatment and insignificantly higher levels when compared to patients with spontaneous resolution of the virus. However, the levels of miR-221 in these two groups were still higher as compared to control.

Many previous researches linked miR-221 to the severity of liver fibrosis.[19,20] Others also demonstrated that circulatory miR-221 represents a novel noninvasive biomarker for the risk of disease progression.[13,20] Higher levels of miR-221 may reflect liver cell damage induced by chronic HCV infection due to activation of hepatic stellate cells (HSCs),[21] implying that circulating miR-221 may reflect the disease activity of HCV infection. Furthermore, the disruption in miR-221 expression level even after HCV clearance might indicate the HCV-induced molecular change. Thus, miR-221 could be used as noninvasive biomarker during long-term follow-up.

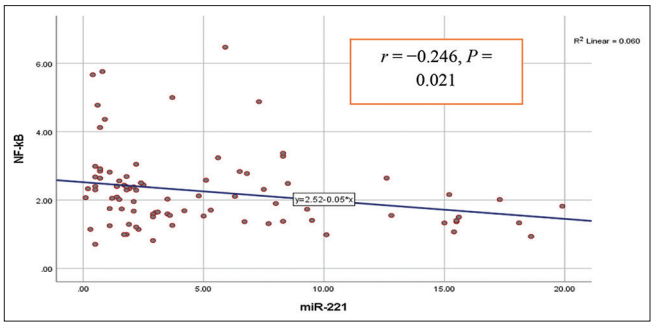

Figure 3: Correlation between miR-221 fold change and NF-kB serum level. miR-221: miRNA-221, NF-kB: Nuclear factor-kappa B

These findings were compatible with those obtained by Mourad *et al*.[22] who stated that the serum level of miR-221 increased in chronic hepatitis C (CHC) patients compared to the healthy control. Another study conducted by Ding *et al*.[20] confirmed these findings. On the other hand, the present results contradict what was reported by previous studies, which showed that the difference in serum miR-221 levels between CHC and healthy control was statistically insignificant.[13,23]

Notoriously, there is a scarcity of information about the immune status and molecular changes in miR-221 expression after spontaneous or treatment-based clearance of the virus. The present investigation is the first to indicate a disturbance in miR-221 expression in HCV-previously exposed individuals that remains even after HCV clearance. Hence, we were unable to validate our findings in comparison with other equivalent researches.

In addition to all of the above, the present study showed that there was a low negative correlation between circulatory miR-221and NF-κB serum level decrease.

It was previously documented that NF-κB is tightly controlled in normal conditions. Nonetheless, in many inflammatory diseases and tumors, molecular alteration results in NF-κB dysregulation.[24] NF-κB demonstrated to influence miRNA expression and vice versa through positive and negative feedback loops. For instance, two separate NF-κB-specific binding sites have been identified in upstream promoter of miR-221, indicating that the expression of miR-221 is induced by NF-κB activation.[25,26] Another study conducted by Ding *et al*.[20] demonstrated that "miR-221 can be induced by HCV infection in an NF-κB-dependent manner which plays an important role in inflammation-associated cancer." Moreover, miR-221 act in a positive feedback loop to increase expression levels of the Rel A component of NF-κB to promote constitutive activation of this factor.[27] These findings, in addition to ours, add to the perception of the processes that drive inflammatory response and point to NF-kB as a possible therapeutic target for HCV infection.

Conclusion

The findings of this investigation show that HCV infection disrupts NF-κB activation, resulting in dysregulation of miR-221 that persists long after the virus has been cleared. Thus, miR-221 could be used as noninvasive biomarker during long-term follow-up. Understanding the NF-κB– miR-221 regulation networks may thus provide potential usage in pharmacology and therapeutic personalization. Furthermore, a miRNAs profile analysis can help distinguish HCV-exposed individuals from healthy controls.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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