# Role of MicroRNA-221 and HCV Genotype in Predicting Chronic Liver Disease among Iraqi Patients

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**Abstract**

MicroRNAs (miRNAs) have been repeatedly shown to play important roles in liver pathologies, including hepatitis, liver cirrhosis, and liver cancer. miRNAs are small RNA molecules that have the ability to regulate gene expression by targeting mRNA degradation or translational repression. miRNA-221 is one of the over 700 kinds of currently known miRNAs and is up-regulated in multiple tumors. This work was carried out to evaluate the ratio of HCV genotype in the studied sample of Iraqi HCV positive patients and to assess the expression level of miRNA-221 in the plasma as a diagnostic marker of liver injury in chronic hepatitis C and (liver cirrhosis & hepatocellular carcinoma). Current study included 25 healthy controls, 35 patients with chronic hepatitis C, 20 patients with (liver cirrhosis and hepatocellular carcinoma) who attended to the Gastroenterology and Hepatology Teaching Hospital in Baghdad during the period from June/2017 to February/2018 were included in this study. HCV Genotype, viral load and miRNA-221 in the plasma were measured using real‑time PCR. This study showed that statistically significant differences in the mean values of miRNA-221 in plasma between the studied groups (P value =0.04) and also observed highly significant fold change in miRNA-221 expression was found between (liver cirrhosis and hepatocellular carcinoma *vs* control) groups. In conclusion measurements of miRNA-221 may be useful in the evaluation of HCV patients. Moreover, the most prevalence genotype in this study was genotype 4 then 1a.

***Keywords***: Chronic Hepatitis C virus, Liver cirrhosis, Hepatocellular carcinoma, Genotype and miRNA-221.

# دورالحمض الريبوزي النووي الدقيق - 221 والنمط الجيني لفيروس HCV في التنبؤ بأمراض الكبد المزمنة بين المرضى العراقيين.

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**الخلاصة**

لقد أثبت الاحماض النووية الريبوزية النووي ميكروي مرارًا وتكرارًا أنها تلعب أدوارًا مهمة في أمراض الكبد ، بما في ذلك التهاب الكبد وتليف الكبد وسرطان الكبد,وهي عبارة عن جزيئات الحمض النووي الريبي الصغيرة التي لديها القدرة على تنظيم التعبير الجيني عن طريق استهداف تحطيم الرنا المرسل أو القمع التعددية . الحمض الريبوزي النووي الميكروي 221 هو واحد من أكثر من 700 نوع من الاحماض النوورية الميكروية المعروفة حاليًا ويتم تنظيمها في أورام متعددة. يهدف هذا العمل تقييم نسبة النمط الوراثي الالتهاب الكبد الفايروسي سي في العينة المدروسة من مرضى التهاب HCV العراقيين وتقييم مستوى التعبير عن الحمض النووي الريبوزي الميكروي 221 في البلازما كعلامة تشخيصية لإصابة الكبد في التهاب الكبد المزمن C و (تليف الكبد و سرطان الكبد). اشتملت الدراسة الحالية على 25 عنصر تحكم صحي ، و 35 مريضًا يعانون من التهاب الكبد المزمن ، و 20مريضًا يعانون من تليف الكبد و سرطان الكبد ، والذين حضروا إلى مستشفى أمراض الجهاز الهضمي والكبد في بغداد خلال الفترة من يونيو / 2017 إلى فبراير / 2018 ، تم تضمينهم في هذه الدراسة. تم قياس النمط الوراثي HCV ، الحمل الفيروسي و miRNA-221 في البلازما باستخدام تفاعل البلمرة التسلسلي الكمي في الوقت الحقيقي. أظهرت هذه الدراسة أن فروق ذات دلالة إحصائية في متوسط قيم miRNA-221 في البلازما بين المجموعات المدروسة (قيمة P = 0.04) ولاحظت أيضًا تغيرًا كبيرًا في في تعبير miRNA-221 بين مجموعات (LC & HCC vs control) . من ناحية أخرى ، لم يكن هناك أي تغيير ذو دلالة إحصائية في مستوى التعبير miR-221 بين مجموعة (CHC vs control) أو مجموعة (CHC vs LC & HCC). وفي الختام ، قد تكون قياس miRNA-221 مفيدة في تقييم مرضى التهاب الكبد الفايروسي سي . علاوة على ذلك ، كان النمط الوراثي الأكثر انتشارًا في هذه الدراسة هو التركيب الوراثي 4 ثم 1 أ.

الكلمات المفتاحية: التهاب الكبد الفيروسي سي المزمن ، تليف الكبد ، سرطان خلايا الكبد ، التركيب الوراثي و الحمض الريبوزي النووي الدقيق.

## Introduction

HCV is a global health issue and the major etiological agent of chronic hepatitis and liver disease worldwide. More than half of case infected with HCV develop CHC, perhaps leading to fibrosis, cirrhosis, end-stage liver disease, and HCC [1]. Many efforts around the world have been devoted to HCV genotyping and detection of serum HCV RNA level [2]. Analysis of the HCV genome shows a remarkable genetic

heterogeneity among HCV isolates from all over the world and the main risk factor is exposure to infected blood or blood products, unsterile needle-sharing among intravenous drug users (IVDU), and needle stick injuries in health care workers [3]. To date, at least six major genotypes of HCV and over 67 different subtypes on the amount of nucleotide variation [4,5]. HCV RNA level is an important seromarker for the diagnosis of active HCV infection and the monitoring of virologic response to antiviral therapy [6].

In recent years microRNAs (miRNAs), a family of short (average of 20~25 nucleotide long), naturally occurring, small a non-coding RNAs have emerged as important post-transcriptional regulators of gene expression. There may be thousands of miRNA genes in the human genome, transcribed by RNA polymerase as long primary miRNA molecules, then processed in the nucleus forming pre-miRNAs. These pre-miRNAs get transported from the nucleus to the cytoplasm for further processing. miRNAs are predicted to control the activity of more than 60% of all protein-coding genes [7]. The miRNAs have different biological functions, such as miRNA-221 (miR-221), one of the miRNAs that be either oncogenes or tumor suppressor genes involved in tumor formation, was confirmed overexpression in HCC tissues while the function and mechanism were not clear at present [8].

The goal of the present study was to evaluate circulating plasma miRNA-221 expression levels in Iraqi patients with HCV as well as to explore their potential as non-invasive markers for diagnosis in chronic liver disease.

## Materials and Methods

### Subject

This study was carried out on 80 patients who were divided into three groups: Group I: included a total of 35 cases (21 males and 14 females) of chronic hepatitis C patients whose age range was (15-70) years. Group II: 20 cases (12 males and 8 females) of (liver cirrhosis & hepatocellular carcinoma) with age range between (28-70) years. Group III: 25 apparently healthy subjects with ages (18-60) years who were negative for HCV and HBV by ELISA.

Participants were enrolled from the Gastroenterology and Hepatology Teaching Hospital and Dowaly Private Hospital in Baghdad during the period from June/2017 to Feb /2018. The clinical diagnosis was based on the decision of physician in the Gastroenterology and Hepatology Teaching Hospital and testing for anti-HCV was repeated in all recruited anti-HCV-positive patients using a third-generation immunoassay that allows the detection of antibodies to the NS3, NS4 and NS5 core antigens of the virus and if this assay gave a positive result, a PCR technique was performed.

Patients were selected if they had no other causes of liver disease, autoimmune or metabolic disorders, HCC or co-infection with hepatitis B virus and/or human immunodeficiency virus, liver steatosis, malignancies, and current alcohol abuse.

**Molecular detection of miRNA-221**

Molecular detection of miRNA‑221 was carried out according to (TaqMan™ MicroRNA Assay, inventoried, SM, Applied Biosystems , USA) which occurred in three steps:

1. RNA extraction.
2. Reverse transcription step.
3. Real‑time PCR for the detection of mature miRNA‑221.

**HCV RNA analysis of hepatitis:**

The amounts of serum HCV RNA were measured by quantitative PCR assay using detection kit (HCV Virus-RG RT-PCR Kit, Sacace).

**Detection the genotypes of HCV:**

HCV Genotype detection according to (plus Real-TM HCV Real-TM Quant Dx, Sacace).

**Statistical analysis**

Analysis of data was carried out using the available statistical package of SPSS-25 (Statistical Packages for Social Sciences- version 25). Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). The significance of difference of different means (quantitative data) were tested using Students-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) were tested using Pearson Chi-square test ($\chi^2$-test) with application of Yate's correction or Fisher Exact test whenever applicable. Statistical significance was considered whenever the P value was equal or less than 0.05.

## Results

Table (1) revealed the demographic distribution of all studied groups according to gender and age were shown .The results showed that there were 21 male (60%) and 14(40 %) female patients with mean age was (39.5) years in the CHC group, while there were 12 male (60%) and 8 female (40 %) patients in the (liver cirrhosis & hepatocellular carcinoma) group with mean age was (52.5) years. Moreover, the control group had 13 male (52 %) and 12 female participants (48%) and the mean age was (35.9) years.

It was shown that the highest percentages of the two studied groups were males. Besides, the standard deviation for the groups was large which showed large variations in the age within groups, most likely due to the large variation of the subjects' ages in this study.

However, results in this table demonstrated that there was no statistically significant difference among the ages and genders of these groups were (P=0.061, 0.798) respectively.

**Table (1):** The baseline characteristics of the studied groups.

| Variables | | Patients | | Control | P-value |
|---|---|---|---|---|---|
| | | CHC | (LC&HCC) | | |
| Age (years) | Range | 15-70 | 28-70 | 18-60 | P=0.061 NS (*) |
| | Mean ± SD | 39.5±16.7 | 52.5±11.6 | 35.9±11.3 | |
| Gender | Male No. (%) | 21(60.0) | 12(60.0) | 13(52.0) | P=0.798 NS (*) |
| | Female No. (%) | 14(40.0) | 8(40.0) | 12(48.0) | |
| Total No. | | 35 | 20 | 25 | |
| *Significant difference between proportions using Pearson Chi-square test at 0.05 | | | | | |

Table 2: Descriptive statistics of viral load by IU/ml in chronic HCV patients according to the baseline characteristics were shown in table (2). The descriptive data based on gender showed that the major viral load value was Mean ($16.73\times10^6\pm5.79\times10^6$) monitored in males which found in 21(60.0%) of the patients, while the less viral load value in the female which made Mean ($12.23\times10^6\pm4.09\times10^6$) found in 14(40.0%) of patients. According to the age group.

The results revealed a higher value in viral load was with the interval age group (30-59) years which found in 16(45.7%) of the patients, with Mean ($15.74\times10^6\pm4.14\times10^6$). while patients at age group (<30) years and, (=>60) years had an equal viral load with Mean± SD were ($14.08\times10^6\pm3.02\times10^6$; $14.71\times10^6\pm3.58\times10^6$) respectively. Moreover, the demographic characteristics of the viral load of this study showed that the p-values of the differences among the ages and genders of these groups were (0.972, 0.491) respectively. These differences were non-significant at P>0.05.

**Table (2):** Distribution of viral load according to demographic characteristics in chronic HCV patients

| | | **Mean ±SD IU\ml** | **No. (%)** | |
|---|---|---|---|---|
| **Gender** | **Male** | $16.73\times10^6\pm5.79\times10^6$ | 21(60.0%) | 0.491 NS |
| | **Female** | $12.23\times10^6\pm4.09\times10^6$ | 14(40.0%) | |
| **Age groups** | **<30 years** | $14.08\times10^6\pm3.02\times10^6$ | 14 (40.0%) | 0.972 NS |
| | **30---59** | $15.74\times10^6\pm4.14\times10^6$ | 16(45.7%) | |
| | **=>60y** | $14.71\times10^6\pm3.58\times10^6$ | 5(14.3%) | |

HCV genotype distribution in fifty-five HCV-positive Iraqi patients as illustrated in figure -1, HCV genotype 4 was the predominant genotype with frequency 19(34.5%) followed by genotype 1 included 1a was 17(30.9%) and 1b was 12(21.8%). The lowest frequency was found in Genotype 2 was 4(7.3%) and Genotype 3 was 3(5.5%).

**Figure 1:** illustrated the frequencies of the genotype in HCV-positive Iraqi patients.

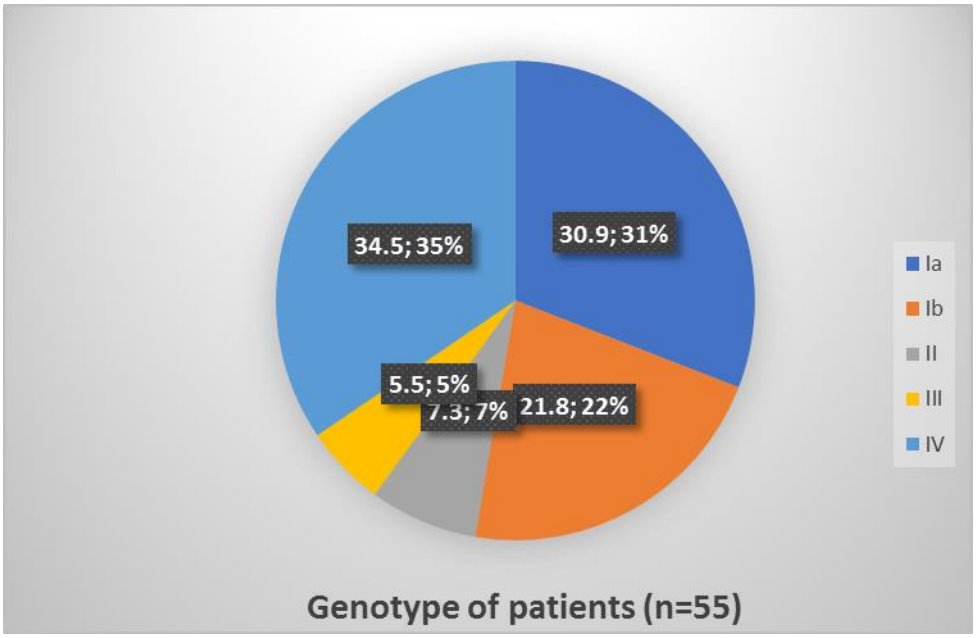


Table 3: the distribution of HCV genotypes & sub genotype in studied groups according to gender. The more frequently observed genotype in males in both groups was genotype 4 with proportion 92.3% (12 out 35) in chronic HCV group and 83.3% (5 out 20) in (liver cirrhosis & hepatocellular carcinoma) group.

There was predominance of Genotype 1a in females than in males in both groups with proportion in chronic HCV was 80.0% (8 out 35) and the proportion was 42.9% (3out 20) in another group, followed by Genotype Ib was [male 5(62.5%) vs female 3(37.5%)] in chronic HCV group and [male 2(50.0%) vs female 2(50.0%)] in (liver cirrhosis & hepatocellular carcinoma) group. Genotype 2 and 3 showed the lowest percentage rate in both genders. This study revealed a non-significant difference in genotype according to gender in both chronic HCV and (liver cirrhosis & hepatocellular carcinoma) groups were (P-value = 0.064, 0.536) respectively.

**Table (3):** Distribution of different HCV genotypes according to gender in the studied groups**.**

| Genotype | Chronic HCV | | | | (LC & HCC) | | | |
|---|---|---|---|---|---|---|---|---|
| | Male | | Female | | Male | | Female | |
| | No | % | No | % | No | % | No | % |
| Ia | 2 | 20.0 | 8 | 80.0 | 4 | 57.1 | 3 | 42.9 |
| Ib | 5 | 62.5 | 3 | 37.5 | 2 | 50.0 | 2 | 50.0 |
| II | 1 | 50.0 | 1 | 50.0 | 1 | 50.0 | 1 | 50.0 |
| III | 1 | 50.0 | 1 | 50.0 | 0 | 0 | 1 | 100 |
| IV | 12 | 92.3 | 1 | 7.7 | 5 | 83.3 | 1 | 16.7 |
| P value | 0.064 | | | | 0.536 | | | |

Table 4: Analysis of mean fold change in expression level of miRNA-221 in patients' plasma in comparison to the healthy control group showed that miRNA-221 displayed significant fold increase expression in chronic HCV group Mean was (6.237) and in (liver cirrhosis & hepatocellular carcinoma) group Mean was (12.193). While, in control group Mean was (3.034). Also, showed statistically significant differences in the mean values of miRNA-221 in plasma between the studied groups (P value =0.04) and observed highly significant fold change in miRNA-221 expression was found between (liver cirrhosis & hepatocellular carcinoma *vs* control) groups. On the other hand, there was no statistically significant fold change in miR-221 expression level between either (CHC vs control) groups or (CHC vs LC&HCC) groups.

**Table (4):** MiRNA-221 plasma levels in studied groups.

| MiRNA-221 | CHC | (LC &HCC) | Control |
|---|---|---|---|
| Mean | 6.237 | 12.193 | 3.034 |
| Standard Error of Mean | 2.008 | 3.764 | 1.047 |
| Median | 1.18 | 8.09 | 0.67 |
| CHC vs Control | 0.212 | | |
| LC&HCC vs Control | 0.014* | | |
| CHC vs LC&HCC | 0.131 | | |
| P comparing all | 0.04# | | |
| *Significant difference between two independent means using Students-t-test at 0.05 level | | | |
| #Significant difference among more than two independent means using ANOVA test at 0.05 level. | | | |

## Discussion

The sex of population in present study was shown that the highest percentages of the studied groups were males. The p-values of the differences among the ages and genders of these groups were (0.061, 0.798) respectively. These differences were non-significant at P>0.05.

The result of this study appeared an agreement with studies done in different Iraqi provinces as AL-Qadisiya and Thi-Qar that observed HCV infections in males was higher than females through epidemiological study [9,10].The results also agree with Al Zayed *et al*.[11] who found that there was non-significant difference demonstrated for gender . But our findings contradict from that of Abdul Hasan,[12] who found equal sex ratio 1:1. This high frequency of infection with HCV among males may be attributed to socio-community nature of Iraqi people which make men undergone the responsibility of working and eventually are in great contact with the pathogens rather than the women. Furthermore, the difference between age groups was failed to reach the levels of statistical significance (P= 0.05). Similar results were

reported by Ezzat *et al*. [7] who showed that the studied groups were age matched ($P > 0.05$) .

However, the observation of present study agrees with previous studies that showed peak prevalence of anti HCV Abs were observed among persons more than 30 years to 49 years [13,14]. This finding was explained generally the high infected patients at age mean (39.5), this may be due to the fact that this group of age has exposed to more accidents, occupational risks, blood transfusion, surgeries, than younger groups of patients. Present results indicated that there were higher HCV RNA levels among males compared to females.

Gender has been rendered a milestone in defining innate tendency to eradicate HCV; the spontaneity with which females abate the HCV load is higher than in males [15]. Premenopausal females are better candidates for interferon responsiveness owing to their lower BMI than men and young age thereby, many women were spared of HCV load despite getting infected [16]. Nevertheless, there is inevitability in the fact that female sex hormones facilitate responsiveness to interferon [17]. Vindictively or naturally testosterone promote expression of scavenger receptors found on human monocyte-derived macrophages and HepG2 cell, receptors on which HCV binds resulting in harbouring higher HCV loads in males than females [18]. Present result was in an agreement with recent studies done by Kareem, [14] which found that HCV RNA levels in patients did not correlate with age or sex. These results were in consistent with finding done in China which has been reported that males have persistent elevated HCV RNA levels and are at higher risk as compared to females [3]. While, our finding conflict with Rong and colleagues who reported that older females had higher HCV viral load value than males [19].

In current study observed that HCV genotype 4 is the predominant genotype followed by 1a and 1b while genotype 3and 2 represent the lowest genotype with absents of detection other genotype 5and 6 in present study. In a study conducted in the south of Iraq, it was found that 35.0% of the samples typed as genotype 4 while 50.0% of the recruited samples typed as genotype 1[20].

Genotype 4 has been linked with increased incidence of cirrhosis and poor response to interferon therapy [21]. HCV genotypes play a crucial rule in assessing therapeutic decisions and approaches. As mentioned above it has shown that the

severity, prognosis of disease and response to therapy may vary according to the HCV genotypes [22]. Additionally, in this study we have studied the distribution of HCV genotypes and associated these genotypes with gender. The results of the current study are clearly divergent from most of other studies done, as that HCV genotype 1 was more found in females while HCV genotype 4 more observed in males and also, there was no variation among the HCV genotypes and gender as different HCV genotypes.

The result of this study also agreement with Abdul Hasan [12] who found that genotype 4 was the dominant in HCV infected male while in female genotype 1a was the common one . A study reported by Khdeir *et al*. [23] showed that there was no significant difference between HCV genotypes and gender were found through a study conducted in Basra province in Iraq from 2014 to 2015 for determine the genotypes of HCV on 102 Basra patients. This observation was matched with recent studies showed that no significant differences in the distribution of HCV genotypes with respect to gender [24,25]. On the other hand, in contrast to our observation in Libya showed that the prevalence of HCV genotype 1 was found to be mostly in males, while genotype 4 was found more frequently in females [26].

The possible explanation for this sex-related difference in HCV genotype distribution among Iraqi patients might be due to low samples size. The deregulation of miRNA expression may be a key factor in many liver diseases including hepatocellular carcinoma. Among the miRNAs that are upregulated in HCC, there is evidence in support of the tumor-promoting activity of miRNA-221. It is upregulated in 70%–80% of HCC samples. From a functional point of view, HCC cells overexpressing miRNA-221 show increased growth, proliferation, migration, and invasion capability [27]. In the current work, there was an increased expression of miRNA -221 in patients compared to healthy controls.

The plasma level of miR-221 was significantly upregulated in (liver cirrhosis & hepatocellular carcinoma) patients compared to healthy control. On the other hand, no significant difference was shown in the expression of miRNA-221 between either (CHC vs control) or (CHC vs LC&HCC) groups.

These results were compatible with that in recent study by Mourad *et al*. [28] who found that the Serum levels of miRNA-221 increase in chronic hepatitis C (CHC) than control ($P < 0.01$). Also, they were observed miRNA-221 were significantly

elevated in the HCC group compared to the control group. Current study came in accordance with previous observations by Zekri *et al*. [29] who demonstrated that the difference in serum miRNA-221 levels between patients with CHC and controls was not statistically significant but inconsistent with our results, they found the expression of miRNA -221 between HCC and control was non-significant (P= 0.15) .

Another study done by Ding who tested serum levels of miRNA-221 and found upregulated in patients with chronic HCV infection compared to healthy blood donors [30]. Also, they found miRNA-221 can be induced by HCV infection in an NF-κB dependent manner. MiR-221 and NF-κB expression were upregulated after HCV infection. This finding suggests that the HCV-induced upregulation of miR-221 requires NF- κB activation and that NF- κB signaling pathway plays an important role in inflammation-associated cancer.

Significant upregulation of miRNA-221 was an event that came in agreement with previous report by Pineau supporting its function as oncogenic miRNA in HCC [31]. MiRNA-221 promotes cell cycle progression through targeting cyclin-dependent kinase inhibitors which are important regulators of G2/M transition, CDKN1B/p27, and CDKN1C/p57 [32]. In addition to the modulation of cell cycle, miRNA-221 contributes to the progression of HCC by the inhibition of apoptosis through targeting a proapoptotic member of the Bcl-2 family BBmf [33].In apparent contrast, Luo *et al*.[34] which indicated that the difference in serum miRNA-221 levels between patients with HCC and controls was not statistically significant. They were suggested that tumor tissue was not the only source of serum miRNA-221. Further studies were needed to investigate the relationship between tumor and serum miRNA-221 levels, and any differences in miRNA-221 levels between different organs to confirm the lack of a diagnostic value of serum miRNA-221 in HCC. The conflicting results between the current study and what has been published in the literature might be attributed to a number of factors that impact the expression pattern of miRNAs in different studies. Such factors include the heterogeneity of patients, treatment, and etiology [35].

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