Circulating microRNA-122 in HCV Cirrhotic Patients with High Frequency of Genotype 3

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Research Article

Keywords: Hepatitis C Virus, Cirrhosis, MicroRNA-122, Genotype 3

DOI: https://doi.org/10.21203/rs.3.rs-701493/v1

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Abstract

MicroRNA-122 (miR-122) is a liver abundant microRNA that is released upon liver injury. In the present study, we investigated the circulating miR-122 profiles in a Pakistani patients’ cohort with HCV chronic liver disease that was mainly based on HCV genotype 3 infections.

From 222 patients with chronic HCV liver disease, classified as mild, moderate, or gross, serum samples were collected. Cell-free RNA was isolated and used for miR-122 quantification by qPCR.

More than 60% of 222 patients were infected with HCV genotype 3. ALT values and HCV viral load showed no correlation with the HCV genotype. Circulating miR-122 levels were significantly upregulated in patients with cirrhosis. Notably, HCV patients with mild cirrhosis showed the most marked increase in serum miR-122 levels (p=0.0001). Furthermore, we proved a positive correlation (r=0.46) of miR-122 with the ALT values in patients with mild cirrhosis.

Importantly, the increased miR-122 profiles of serum samples obtained from patients with mild, moderate, and gross cirrhosis did not depend on the HCV genotype. Importantly, our findings confirm that serum miR-122 levels are significantly upregulated in the HCV cirrhotic patients serving in particular as a biomarker for the non-advanced stages of cirrhosis, independently of the HCV genotype.

Background

Hepatitis C virus (HCV) is in the hepacivirus genus of the Flaviviridae family, with 9.6 kb single-stranded RNA having codons for 3010 amino acids. HCV infection progresses with a high frequency to cirrhosis, and finally hepatocellular carcinoma (HCC). Liver cirrhosis manifests in structural deterioration of the liver tissues due to necrosis and the mortality rate tenth highest in Pakistan and worldwide\(^1\),\(^2\). Around 170 to 200 million patients suffer worldwide of HCV infection of which 2.7 to 3.9 million are in the US\(^3\). According to the World Health Organization (WHO), about 1.412.000 deaths (796.000 due to cirrhosis and 616.000 due to liver carcinoma) are caused by HCV\(^4\)–\(^6\). Until now, there is no vaccine against HCV\(^7\).

Nearly 98% of the untranslated human genome is transcribed into non-coding RNAs (ncRNAs). These ncRNAs are long, short-chain, and do not translate into proteins. The 21–23 nucleotides long ncRNAs and short-chain are called microRNAs (miRNAs). miRNAs are the least mutating and most well-studied type of ncRNAs\(^8\). These miRNAs are present in almost all organisms, controlling gene expression of approximately 30% genes\(^9\), including cancer-related integrin genes\(^10\),\(^11\). Due to this gene regulation ability, the miRNAs play a significant role in cell differentiation, programmed cell death, embryonic development, and cancer control, etc.\(^12\)–\(^14\). These miRNAs can base pairs within the organism as well as with the RNAs of the invading pathogens\(^15\),\(^16\). MicroRNA-122 (miR-122) is known for complementary base pairing with the 5-UTR region of the HCV RNA, promoting viral RNA replication in hepatocytes\(^17\). MicroRNA-122 is highly abundant in the liver, affecting lipid metabolism, gene expression, and insulin resistance\(^18\). Since miR-122 triggers HCV replication by different mechanisms such as enhancing HCV RNA translation and
protecting HCV genome RNA degradation\textsuperscript{18,19}, its inhibition is a promising therapeutic approach in recent HCV treatment concepts\textsuperscript{19}.

Presently, there is only limited information about miR-122 profiles in HCV cohorts with the high frequency of genotype 3. HCV genotype 3 infections are prominently occurring in the Asian population\textsuperscript{20}. Patients with chronic HCV genotype 3 infections suffer from fast progression of fibrogenesis and a high incidence of hepatocellular carcinoma (HCC)\textsuperscript{21}. Furthermore, HCV genotype 3 frequently leads to a change in lipid metabolism\textsuperscript{22} and severe steatosis\textsuperscript{23}.

**Patients And Methods**

**Ethical approval**

The study was approved by the Ethical Committee, Center of Biotechnology & Microbiology (COBAM), University of Peshawar, Pakistan, all experiments were performed in accordance with relevant named guidelines and regulations, and written informed consent was obtained from all the healthy controls and recruited patients, whose data containing demographic, clinical characteristics, and estimated infection time and miR-122 levels were used. Since no patients

**Patients**

Samples were collected from the tertiary care hospital of Peshawar (Pakistan) along with demographic data of the patients (Table 1) living in the region of Khyber Pakhtunkhwa, Pakistan. Identification of HCV 5’ UTR and genotyping were performed as previously described by the procedure of Ullah \textit{et al.}\textsuperscript{1}.

**RNA isolation**

Blood samples of HCV cirrhotic patients were collected, centrifuged at 3.000 rpm for 10 minutes at 4°C, and stored at -80 °C. RNA was extracted from the serum samples using the manufacturing protocol of Qiagen Kit (Qiagen, Hilden, Germany. Cat No./ID: 217204). SV40-miRNA (Qiagen) was transferred to serum samples (2 pmol/200 ml) before the RNA extraction method for later normalization of miR-122 levels.

**cDNA and Real-Time PCR**

MicroRNA was analyzed by a two-step real-time PCR and complementary DNA was prepared by the miScript-Reverse Transcription Kit (Qiagen kit. Cat No./ID: 218160) and the miR-SYBR Green PCR Kit (Qiagen, Hilden, Germany. Cat No./ID: 218073). The miR-122 and SV-40 primers were used for cDNA synthesis and cycling protocol followed was; 37 °C / 60 min, 95 °C / 10 min and 22 °C /
Thermo cycling conditions of the RT-PCR were as follows: initial denaturation 95 °C/3 min, following 49 cycles of PCR template denaturation 94 °C / 30 sec, annealing 55 °C / 45 sec and extension 70 °C / 45 sec. The melting curve analysis was done at 50-95 °C / 0.5 sec. All the steps were done in triplicate and according to the supplier's guidelines. Spike-in SV40-miRNA (Qiagen, Hilden, Germany. Cat No./ID: 331535) was used for normalization of extracellular miR-122 levels.

**Statistical Analysis**

Statistical analysis was performed in IBM SPSS software 25.0 and GraphPad Prism 5. The mean, standard deviation, ranges different other variables are reported in Table 1. A one-way ANOVA test was performed for the statistical significance between the groups. Student test (t-test) was performed for the significance between two variables. The significance of ROC, AUC curve, sensitivity, specificity, positive and negative values were studied for diagnostic precision of miR-122. Correlation (Pearson Correlation = r) was performed for two variables that are linearly correlated. The values which are less than 0.05 were considered to be statistically significant (p).

**Results**

**Characteristics of patients**

The HCV cirrhotic patients, who agreed to the study, were admitted to the tertiary hospitals Peshawar from April 2016 to October 2018. The demographic summary and clinical information of the patients are shown in Table 1. The HCV cirrhotic patients were categorized into three groups of mild, moderate, and gross cirrhosis. The frequency of patients with gross cirrhosis (n=101) was higher than those suffering from moderate (n=87) and mild (n=37) cirrhosis (Figure 1 A, B). The mean age of the patients was 49 ± 12 years. Patients, aged 31 to 50 years, reported the highest frequency with gross cirrhosis (Figure 1 C). Furthermore, there were significant differences of the alanine transaminase (ALT) and alpha-fetoprotein (AFP) values between the patient groups with no cirrhosis or mild, moderate, and gross cirrhosis, respectively (p< 0.0001 c.f. Table 1). Genotype 3 was the most frequent in the studied cohort (Figure 2) because the samples were collected from patients living in the region of Khyber Pakhtunkhwa in Pakistan, where the HCV genotype 3 is the most frequent HCV genotype of chronic HCV hepatitis (Figure 2). There was no difference observed in the viral load of HCV genotype 3 and non-genotype 3 infections (Figure 3 A). Furthermore, the ALT values which are increasing with the progression of chronic HCV hepatitis, showing the highest levels in patients with gross cirrhosis, were equally distributed in patients infected with the HCV genotype 3 versus patients with non-genotype 3 HCV genotypes (Figure 3 B).
| Parameters      | Patients |
|----------------|----------|
| **Gender**     |          |
| Male           | 136 (61%)|
| Female         | 86 (39%) |
| **Age**        |          |
| mean± standard deviation (SD) years | 49.03± 12.65 |
| mode           | 50       |
| **Cirrhosis**  |          |
| Mild (n)       | 37       |
| mean± SD years | 50.56± 13.19 |
| mode           | 50       |
| Moderate (n)   | 87       |
| mean± (SD years | 47.18± 12.25 |
| mode           | 55       |
| Gross (n)      | 101      |
| mean± (SD years | 48.0± 11.94 |
| mode           | 50       |
| **AST**        |          |
| mean± standard deviation | 121.29± 28.01 |
| Mode           | 122      |
| Significant differences | p< 0.0001 |
| **ALT**        |          |
| mean± SD       | 121.40± 29.03 |
| mode           | 139      |
| mild(mean± SD) | 90.05± 17.11 |
| moderate(mean± SD) | 112.57± 23.59 |
| gross(mean± SD) | 140.24± 22.40 |
| significant differences | p< 0.0001 |
| **AFP**        |          |
| mean± standard deviation | 71.05± 57.09 |
| mode                  | 40       |
|----------------------|----------|
| mild(mean± SD)        | 32.59± 7.77 |
| moderate(mean± SD)    | 35.51± 5.58 |
| gross(mean± SD)       | 114.54± 60.37 |
| significant differences| p< 0.0001 |

| Bilirubin mg/dl       |         |
|-----------------------|----------|
| mean± standard deviation| 1.3±0.6 |

| Size of spleen        |         |
|-----------------------|----------|
| Enlarge (n)           | 117(52.70%) |
| Normal (n)            | 105(47.29%) |

| Blood pressure        |         |
|-----------------------|----------|
| High (n)              | 51(22.97%) |
| Low (n)               | 91(40.99%) |
| Normal (n)            | 80(36.03%) |

| Ascites               |         |
|-----------------------|----------|
| Less (n)              | 26(11.71%) |
| Moderate (n)          | 90(40.54%) |
| High (n)              | 106(47.74%) |

| Respiratory system    |         |
|-----------------------|----------|
| Normal (n)            | 206(92.79%) |
| Abnormal (n)          | 16(7.20%) |

| Cardiovascular        |         |
|-----------------------|----------|
| Normal                | 108(48.64%) |
| Low                   | 16(7.20%) |
| High                  | 101(45.49%) |

Abbreviations: ALT: alaninamino transferase, AST: aspartate aminotransferase, AFP: alpha fetoprotein, SD: standard deviation

**Expression of miR-122 in HCV cirrhotic patients**
MicroRNA-122 expression levels were quantified by qRT-PCR and the expression of the control group was compared with the mild, moderate, and gross patients (Figure 4 A). In evaluation of hepatic miR-122, it was observed that circulating miR-122 was significantly upregulated in the HCV cirrhotic patients compared with control (ANOVA \( p = 0.0019 \)) as shown in Figure 4 and Supplemental Figure 1 A. The comparison of the miR-122 levels in the control patients with the serum miR-122 values of patients with mild, moderate, and gross cirrhosis showed a significant upregulation (\( \text{control vs mild} = 0.0001, \text{control vs moderate} = 0.0032, \text{control vs gross} = 0.0001 \)) (Supplemental Figure 1 A, B, C, Supplemental Table 1).

The comparison of the miR-122 levels with the ALT and AFP values revealed that there were no significant positive correlation. The significant difference was also shown by Tukey's Multiple Comparison Test as well as Bartlett's test which was performed to show significant equal variances in the HCV cirrhotic groups (Supplemental Table 1).

Next, we performed the analysis of receiver operating characteristics (ROC). The area under the curve (AUC) revealed the potential of miR-122 to discriminate between healthy blood donors and patients with chronic HCV liver disease (Figure 5, Supplemental Table 2). Though miR-122 levels efficiently indicate moderate and gross cirrhosis, the sensitivity of increased miR-122 levels was highest to prove in mild HCV cirrhosis (Figure 5 A-C). Also, the ROC analysis showed that there was no difference between non-genotype 3 and genotype 3 based HCV hepatitis (data not shown). Figure 5 D indicates the collective significance of ROC curves.

**Discussion**

MicroRNAs are becoming the accurate and non-invasive biomarkers of diagnosis for hepatic disease soon. Currently, there are no such diagnostic markers to detect liver diseases in their initial stages. The role of miRNAs in the regulation of HCV through modification of the host genes expression has been established recently\(^{17,24,25} \). In the present study, a total of 222 liver cirrhotic patients from the Khyber Pakhtunkhwa, Pakistan, were classified according to mild, moderate, and gross cirrhosis. Genotype 3 (a, b) is the predominant genotype recorded in Pakistan as in most Asian regions\(^{20} \). HCV genotype 3 was reported to occur particularly in patients with gross cirrhosis that have raised higher ALT levels as compared to patients with mild and moderate cirrhosis. This is in agreement with previous findings, describing more severe cirrhosis progression in patients with HCV genotype 3 infections\(^{21} \).

As we and others have shown by previous studies\(^{26-29} \), overall the miR-122 was highly expressed and significantly up-regulated (\( p = 0.0001 \)) in HCV patients as compared to the healthy blood donors. Importantly, circulating miR-122 levels were highest in patients with beginning mild cirrhosis, whereas levels in serum samples from patients with more advanced cirrhosis were only moderately increased confirming previous findings on HCV cohorts, most frequently infected with genotype 1\(^{21} \). The comparison of the miR-122 pattern between genotype 3 and non-genotype 3 HCV infected patients with mild, moderate, and gross cirrhosis proved that the pronounced increase of circulating miR-122 was also observed in the patient cohort infected with HCV genotype 3. Thus, the marked increase of serum miR-
122 levels in the beginning stage of cirrhosis is not dependent on the genotype. Oliveira et al. also found no difference in the increase of circulating miR-122 levels between serum samples of genotype 1 and 3 patients, whereas in liver biopsies the miR-122 changes were more pronounced upon genotype 3 than genotype 1 HCV infection.  

MicroRNA-122 levels in healthy controls were compared with the three groups using Pearson correlation. The gross patients revealed significant positive correlation (r= 0.46, p=0.006). Interestingly, based on patient groups, the analysis of the miR-122 indicated that gross patients were severely affected as compared to moderate and mild. Similarly, the patient groups were also evaluated and correlated with alanine amino-transaminase (ALT) levels. The gross patients had a high level of ALTs as compared to the moderate and mild groups. Besides this, the other two groups (mild and moderate) of miR-122 demonstrate a negligible correlation with ALT. This data is in agreement with the previous findings of Bihrer et al. who reported that the high levels of miR-122 in serum samples obtained from patients with chronic hepatitis C were associated with ALT activities, necro-inflammatory activity, and levels of ALT.

Another remarkable study of the research work was the ROC, AUC, sensitivity, and positivity. The sensitivity rate of miR-122 was significant in the HCV cirrhotic patients. The study further describes that comparison of healthy control with mild patients’ area under the ROC curve (0.8476) was significant (p=0.0001, 95% confidence interval 0.7359 to 0.9593) sensitivity rate as compared to moderate and gross group. There is no true positive rate reported between moderate and gross patients and hence no significant ROC and area AUC was found. The overall result of the miR-122 area under the ROC curve was significantly true positive. The above consequences reveal the clinical potential of the serum miRNA panel, with enhanced specificity and sensitivity in the HCV cirrhotic patients.

The study of biochemical parameters, AFP and ALT, were compared with serum miR-122 to determine the level of specificity and sensitivity. The AFP display no significant sensitivity results with miR-122 and the ALT was reported a significantly positive ROC curve with mild, moderate, and gross patients. Therefore, all the above outcomes of our work are closely related to the study of Weis and Butt et al.

In conclusion, miR-122 has a vital role in HCV replication and significantly expressed in HCV cirrhotic patients as compared to healthy controls. Notably, our data demonstrate that the increased serum miR-122 levels, indicating sensitively hepatic disease progression, do not depend on the HCV genotype though genotype 3 HCV infection is known to be associated with more severe liver cirrhosis and steatosis.

**Abbreviations**

AFP: alpha-fetoprotein, ALT: alanine transaminase, AST: aspartate aminotransferase, AUC: area under curve, HCC: hepatocellular carcinoma, HCV: hepatitis C virus, miR-122: microRNA-122, ROC: receiver operating characteristic, SPSS: statistical package for social science, SVR: sustained virological response.
Declarations

ETHICAL COMMITTEE APPROVAL

Ethical approval and informed consent were obtained from the Ethics Committee of the Center of Biotechnology and Microbiology, University of Peshawar.

CONFLICT OF INTEREST DECLARATION & CONSENT FOR PUBLICATION

All authors confirm that there is no potential financial or any other conflict of interest and all authors give their consent for publication.

STATEMENT OF FINANCIAL SUPPORT AND DISCLOSURE STATEMENT

All authors confirm that they have no financial or commercial interests. The project was funded by the Higher Education Commission (HEC) of Pakistan.

AUTHORS CONTRIBUTION

AU performed the data collection, statistical analysis, and wrote the primary version of the manuscript. XY was involved in RNA isolation and MO designed the overall experiments. SM, BA, IR, JA, QA and TN contributed in discussions, reviewing and in data presentation. All co-authors provided critical revisions and agreed to the final draft. MO was the main supervisor of the project.

ACKNOWLEDGMENT

We are very thankful to the tertiary care hospitals of Peshawar, Pakistan for helping us in collecting the samples and the data. Furthermore, we appreciate the technical support of Ulrike Koitzsch and Hannah Eischeidt-Scholz (Institute for Pathology; University Hospital Cologne, Germany), who assisted in RNA isolation and qPCR quantification. The authors are very grateful to the Higher Education Commission (HEC) of Pakistan for supporting Amin Ullah with an IRSIP fellowship.

Confirmation and Consent to publish:

All authors confirmed that there was no conflict of interest and gave their consent for publication. Because there are no data in the manuscript that reveal the identity of the patients, patients´ consent
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**Figures**
Figure 1

A: Distribution of chronically HCV infected patients suffering from mild, moderate, or gross cirrhosis. B: Distribution of the HCV infected patients in respect to their cirrhosis stage (mild, moderate, and gross). C: Age distribution of HCV cirrhotic patients.
Figure 2

Distribution of HCV genotypes (1b, 2a, 3a, 3b, mixed and untyped) in the patients with chronic HCV hepatitis.
Figure 3

Comparison of Non-Genotype 3 vs. Genotype 3 in patients with mild, moderate and gross cirrhosis. A: HCV, B: ALT.
Figure 4

Expression levels of miR-122 in HCV cirrhotic patients. A: shows the miR-122 levels in the control group and patients with mild, moderate, and gross cirrhosis. The miR-122 levels were significantly upregulated in the HCV group with mild, moderate, and gross cirrhosis. The data of ΔCq of the normalized expression miR-122 values are presented as Whisker box plots. B: Comparison of expression levels of miR-122 in Genotype 3 (in blue) vs. Non-Genotype 3 (in red).
Figure 5

Receiver Operating Characteristic (ROC) curve of serum miR-122 in the HCV patients. ROC curves have been drawn and AUC curves were used to assess and examine the diagnostic ability of miR-122. A-C: The miR-122 healthy control was compared to HCV cirrhotic patients (mild, moderate, and gross group). D: shows the combined ROC curve of the HCV patients’ groups.

Supplementary Files
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- SupplementalTables.docx