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Evaluation of miR-122 Level in the Plasma of Chronically HCV Infected Patients[1]

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Abstract—MicroRNAs (miRNAs) are small non-coding RNA molecules, which have an important function in regulating RNA stability and gene expression. They also can circulate in a cell-free form in the blood that-makes them potential disease markers. The liver contains various classes of miRNAs in which miR-122 accounts for about 70% of all miRNAs and it has been proved that its level increases in case of liver damage. Here, we investigated plasma levels of miR-122 as a useful disease parameter in patients with chronic hepatitis C (CHC) infection. Thirty five hemophilia and thalassemia patients with CHC were studied. The total RNA was extracted from plasma samples, and miR-122 levels were measured by qPCR and then compared with the specific liver markers. The plasma levels of alanine transaminase (ALT) and aspartate transaminase(AST) were correlated with plasma miR-122 level in CHC patients, and the level of circulating miR-122 in healthy individual groups were rarely lower than those of patients with CHC. In our study, miR-122 levels correlated well with markers of liver inflammatory activity. Plasma miR-122 can be assumed to be another marker in liver similar to the currently used specific markers such as ALT and AST for evaluation of liver damage in hepatitis C virus (HCV) infected patients. Moreover, the correlation between miR-122 and ALT was shown to be higher than between miR-122 and AST.

Keywords: microRNA, hepatitis C virus, liver, ALT, AST
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About 170 million patients around the world are chronically infected with the hepatitis C virus (HCV) [1]. Chronic HCV (CHC) infection can result in severe liver diseases such as cirrhosisand hepatocellular carcinoma in a significant portion of patients after several years of being infected. Eradication of the virus is the primary goal for the inhibition of CHC progression [2]. The most widely-used biochemical markers for estimating hepatocellular damage are assessing enzymatic activities of alanine transaminase (ALT) and aspartate transaminase (AST) in the blood. It should be noted that plasma ALT and AST activities are increased in some other clinical disorders besides liver diseases [3, 4]. These biomarkers do not always correlate well with histomorphological data in clinical applications [1]. Studies have attempted to identify more specific and reliable markers of liver injury to supplement the information provided by ALT or AST activities [1]. Although, the progress has not led to the development of any markers that are better than ALT or AST, microRNAs (miRNAs) have emerged as new potential markers for assessment of liver disease in patients with chronic viral hepatitis [2, 3].

MiRNAs are a class of small non-coding RNAs, 18–25 nucleotides in length that can degrade or block target mRNAs at the posttranscriptional level [4]. MiRNA types that change in tissue might correlate with certain disease states [5].Increasing amounts of data support the idea that cells can actively secrete miRNAs into the circulation system *via* the exosomes. Exosomesare 30–90 nm vesicles secreted by a wide range of mammalian cell types which release their contents outside of its cell of origin. The contents are released when multivesicular bodies formthe endosomes fuse with the plasma membrane [6, 7]. MiRNAs exist in peripheral blood in a remarkably stable form; consequently, they might serve as potential blood-based biomarkers [8]. Cell-free miRNAs in the body fluids are stable under harsh conditions including boiling, low/high pH, extended storage and multiple

[1] The article is published in the original.

Table 1. The characteristics of patients enrolled in the study

Parameters	Value, CHC-patients[a]/control group[b]		
	minimal	maximal	mean
Age, years	16/18	49/42	30/28
ALT, IU/L	12.00/12.00	262.00/28.00	52.51/14.25
AST, IU/L	12.00/12.00	208.00/26.00	40.94/13.50
Anti-HCV-antibody	+/–		
Anti-HIV-antibody	–/–		
Anti-HBV-antibody	–/–		

[a] The group of patients with CHC included 28 men and 7 women infected with HCV genotypes 1a, 1b or 3. [b] The control group included 28 men and 7 women.

freeze-thaw cycles [9]. Synthetic mRNA was found to be quickly degraded bythe high levels of RNAse activity in plasma [10]. MiRNAs can be used as potential biomarkers of drug-induced liverinjury in animal models [11]. The predominant miRNA in the liver is miR-122, which accounts for about 70% of all miRNAs in the liver [2, 12]. The liver contains a large amount of miR-122, while other tissues contain only asmall amount of this miRNA [13]. MiRNAs were also found to circulate in the blood in a cell-free formthat makes them a potential and readily accessible disease markers. Recently it has been found that plasma miR-122 levels are elevated in the treatment-naive CHC carriers with signs of liver damage [2]. Hepatic miR-122 levels have been described as predictive markers for antiviral therapy in CHC patients and inpatients who had not respondedto the antiviral combination therapywith pegylated interferon (PEG-IFN) and ribavirin it was observed that miR-122 levels were decreased in the tissue samples of liver biopsies. The blood level of miR-122 correlates strongly with hepatic necroinflammation; however, the levels of miR-122 in hepatocytes and plasma are not significantly correlated [2]. Here, we studied levels of the liver specific miR-122 in the blood of CHC patients under PEG-IFN and ribavirin treatment.

Ethics statement. This study was approved by the Institutional Ethics Review Board. Volunteers from whom specimens were obtained provided a written in formed consent.

Sample collection. In the presented study 35 CHC patients with confirmed serologic results against HCV and being treated with PEG-INF and ribavirin were investigated. The mean age was 30 years old. A control group of 28 men and 7 women that had neither hepatitis B virus (HBV) nor HIV infection was enrolled in the study. Peripheral blood samples were collected in plasma tubes and supplemented with citrate anticoagulants. The genotypes of HCV in the patients group were 1a, 1b and 3. Inno-LIPA and real-time PCR were used for genotyping and RNA viral load measurements. Viral RNA could be detected in 8 patients that were under PEG-INF and ribavirin treatment. Each blood sample was centrifuged at 3000*g* for 7 min at 4°C for a complete removal of cellular components, and the supernatants were kept at –70°C. Clinical and demographic characteristics of the patients are shown in Table 1.

RNA extraction and reverse transcription. RNA was extracted from plasma samples using the TRIZOL reagent (Invitrogen, USA). Briefly, 1.00 mL of TRIZOL was added to 250 μL of plasma supernatant according to the manufacturer's instructions. To improve precipitation of small RNAs, the supernatant from the chloroform extraction was mixed with an equal volume of absolute ethanol (Merk Inc., Germany) and stored at –80°C overnight. The precipitate was then recovered by centrifugation at 12000*g* at 4°C for 30 min and washed with ice-cold 70% ethanol. After solubilization in RNase-free double distilled water, the purity of isolated RNA was determined by Nanodrop (Biowall Inc., USA), which had showed acceptable and high purity and the OD ratio was $OD_{260}/OD_{280} = 2.0$. Each sample was first treated with DNase to remove traces of DNA impurities, and then polyadenylation and reverse transcription were performed to synthesize cDNA by polyA-polymerase and a specific primer (Parsgengan Co., Iran). The cDNA product was diluted with RNase/DNase-free doubly distilled water (1 : 20) and stored at –80°C.

Quantification of plasma miR-122. qPCR was used for the quantification of miRNAs with an ABI StepOne Sequence Detection System using SYBR Green PCR Master Mix (Applied Biosystems). Melting curve analysis was performed at the end of the PCR cycle in order to validate the specificity of the expected PCR product. Blank controls contained no cDNA. The extracellular miR-122 levels in plasma were normalized to 5S rRNA [14].

We have studied patients infected with HCV of genotypes 1a, 1 or 3 according to the notation in their records. Demographic information is shown in Table 1. Patients with high levels of ALT and AST also showed a significant raise in miR-122 level as it was measured

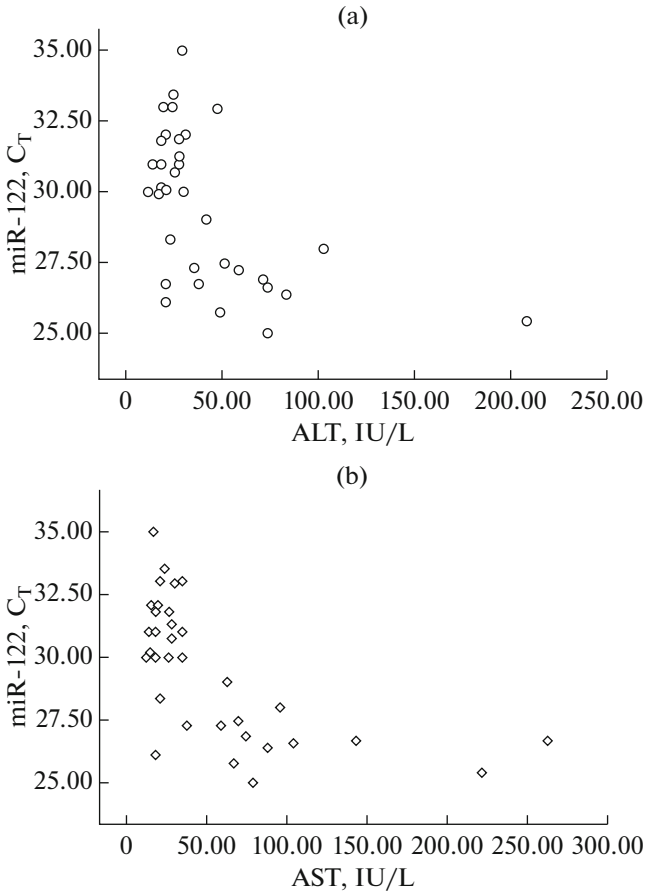

Fig. 1. Correlation between plasma miR-122 levels (C_T) with ALT (a) and AST (b) in HCV infected patients. Points represent the cycle threshold (C_T) of miR-122.

by real-time PCR technique. 5S rRNA which was used as the internal control is expressed ubiquitously. The correlation between the level of miR-122 in plasma and the level of ALT was 0.629 with a confidence interval of 95% (PASW Statistics 18), the correlation with plasma levels of AST was 0.560 with a confidence interval 95%. It was concluded that the plasma level of miR-122 is positively correlated with ALT and AST (Fig. 1).

Earlier studies have revealed that technical and qualitative variation of data normalization for a plasma sample can be controversial. We have used 5S rRNAas the internal control for normalization of the results (REST 2009 Software) and to calculatethe $\Delta\Delta C_T$ between the HCV-infected patients and the negative control group (Table 2). It was shown that the plasma level of miR-122 was up-regulated in samples of HCV infected patients as compared to those from the control group.

Our study suggested that cell-free miRNAs released from pathologically altered tissues can be considered as a useful blood-based biomarker for diagnosis [6, 15, 16]. In contrast to the majority of miRNAs, miR-122 is exclusively expressed in liver which makes this miRNA particularly interesting as a possible biomarker of liver diseases [2]. It was previously shown that the level of miR-122 in blood plasma increases earlier than ALT upon toxic liver injury [17, 18]. Another study showed that carbon tetrachloride-induced acute toxic liver injury leads to an elevation in the plasma miR-122 concentration in rats, and additional reports revealed a correlation between plasma miR-122 and necroinflamatory activity in the liver of patients with chronic HBV infection [2, 12]. These findings indicate that the plasma level of miR-122 can reflect the degree of liver injury; miR-122 may leak from damaged hepatocytes or may be actively exported from altered tissues as a mechanism of adaptation to alterations of the state of a cell. To explore the potential suitability of the plasma level of miR-122 as a disease parameter in HCV infection, the level of miR-122 was compared to other liver parameters that are routinely used to evaluate liver function. It was assessed that level of miR-122 in patients with high level of ALT and AST increased in comparison with the samples from healthy patients. This finding is consistent with some previous studies [19]. Bihrer et al. [2] concluded that miR-122expression was up-regulated in infected patients compared to control group by a mean of 18.087 (SE range 0.665–526.250) [12]. As stated in some other studies, some patients had a high level of ALT and AST while the level of miR-122 did not differ from the healthy group [19]. In the current study the first clinical monitoring was carried out for evaluating miR-122 levels as a possible biomarker for detection of CHC among Iranian patients.

Table 2. Calculation of $\Delta\Delta C_T$ for the evaluation of the difference in miR-122 expression between HCV-positive and control groups (REST 2009 Software*)

Gene	Relative expression level	SE	95% CI	$2^{-\Delta\Delta C_T}$	Conclusion
miR-122	18.087	0.665–526.250	0.069–6.627.511	0.044	Increased expression

* The REST Software calculated data: (1) The ΔC_T value between the target gene (*miR-122*) and the reference gene (*5S rRNA*) was calculated for each sample from HCV-infected (HCV+) or HCV-negative (HCV–) patient: $\Delta C_T = \Delta C_T(miR\text{-}122) - \Delta C_T$ (*5S rRNA*); (2) The difference between the ΔC_T values for HCV+ and HCV– patients was calculated, giving the $\Delta\Delta C_T$ value: $\Delta\Delta C_T = \Delta C_T(HCV+) - \Delta C_T(HCV–)$; 3) Normalized amount of miR-122 was equal to $2^{-\Delta\Delta C_T}$ and the obtained value was used for comparing the two groups.

The serum level of miR-122 seems to relatively correlate with serum ALT and AST release in patients with CHC. Overexpression of miR-122 in samples of HCV infected patients was detected in comparison with miR-122 levels in samples of the control group although the difference rarely was statically significant (p-value = 0.044).

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REFERENCES

1. Williams A.E. 2008. Functional aspects of animal microRNAs. *Cell. Mol. Life Sci.* **65**, 545–562.
2. Bihrer V., Friedrich-Rust M., Kronenberge B., Forestie N., Haupentha J., Shi Y., Peveling-Oberha J., Radek H.H., Sarrazi C., Herrman E., Zeuze S., Waidman O., Piiper A. 2011. Serum miR-122 as a biomarker of necroinflammation in patients with chronic hepatitis C virus infection. *Am. J. Gastroenterol.* **106**, 1663–1669.
3. Iorio M.V., Croce C.M. 2009. MicroRNAs in cancer: Small molecules with a huge impact. *J. Clin. Oncol.* **27**, 5848–5856.
4. Lee Y., Ahn C., Han J., Choi H., Kim J., Yim J., Lee J., Provost P., Radmark O., Kim S., Kim V.N. 2003. The nuclear RNase III Drosha initiates microRNA processing. *Nature.* **425**, 415–419.
5. Ambros V. 2004. The functions of animal microRNAs. *Nature.* **431**, 350–355.
6. Cortez M.A., Bueso-Ramos C., Ferdin J., Lopez-Berestein G., Sood A.K., Calin G.A. 2011. MicroRNAs in body fluids: The mix of hormones and biomarkers. *Nat. Rev. Clin. Oncol.* **8**, 467–477.
7. Wang G.K., Zhu J.Q., Zhang J.T., Li Q., Li Y., He J., Qin Y.W., Jing Q. 2010. Circulating microRNA: A novel potential biomarker for early diagnosis of acute myocardial infarction in humans. *Eur. Heart J.* **31**, 659–666.
8. Chen X., Ba Y., Ma L., Cai X., Yin Y., Wang K., Guo J., Zhang Y., Chen J., Guo X., Li Q., Li X., Wang W., Zhang Y., Wang J., et al. 2008. Characterization of microRNAs in serum: A novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res.* **18**, 997–1006.
9. Mitchell P.S., Parkin R.K., Kroh E.M., Fritz B.R., Wyman S.K., Pogosova-Agadjanyan E.L., Peterson A., Noteboom J., O'Briant K.C., Allen A., Lin D.W., Urban N., Drescher C.W., Knudsen B.S., Stirewalt D.L., et al. 2008. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 10513–10518.
10. Laterza O.F., Lim L., Garrett-Engele P.W., Vlasakova K., Muniappa N., Tanaka W.K., Johnson J.M., Sina J.F., Fare T.L., Sistare F.D., Glaab W.E. 2009. Plasma microRNAs as sensitive and specific biomarkers of tissue injury. *Clin. Chem.* **55**, 1977–1983.
11. Chang J., Nicolas E., Marks D., Sander C., Lerro A., Buendia M.A., Xu C., Mason W.S., Moloshok T., Bort R., Zaret K.S., Taylor J.M. 2004. miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. *RNA Biol.* **1**, 106–113.
12. Bihrer V., Friedrich-Rust M., Kronenberger B., Forestier N., Haupenthal J., Shi Y., Peveling-Oberhag J., Radeke H.H., Sarrazin C., Herrmann E. 2011. Serum miR-122 as a biomarker of necroinflammation in patients with chronic hepatitis C virus infection. *Am. J. Gastroenterol.* **106**, 1663–1669.
13. Sarasin-Filipowicz M., Krol J., Markiewicz I., Heim M.H., Filipowicz W. 2009. Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy. *Nat. Med.* **15**, 31–33.
14. Wang Y., Tang N., Hui T., Wang S., Zeng X., Li H., Ma J. 2013. Identification of endogenous reference genes for RT-qPCR analysis of plasma microRNAs levels in rats with acetaminophen-induced hepatotoxicity. *J. Appl. Toxicol.* **33**, 1330–1336.
15. Zhang J., Zhao H., Gao Y., Zhang W. 2012. Secretory miRNAs as novel cancer biomarkers. *Biochim. Biophys. Acta.* **1826**, 32–43.
16. Starkey Lewis P.J., Dear J., Platt V., Simpson K.J., Craig D.G., Antoine D.J., French N.S., Dhaun N., Webb D.J., Costello E.M. 2011. Circulating microRNAs as potential markers of human drug-induced liver injury. *Hepatology.* **54**, 1767–1776.
17. Wang K., Zhang S., Marzolf B., Troisch P., Brightman A., Hu Z., Hood L.E., Galas D.J. 2009. Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 4402–4407.
18. Zhang Y., Jia Y., Zheng R., Guo Y., Wang Y., Guo H., Fei M., Sun S. 2010. Plasma microRNA-122 as a biomarker for viral-, alcohol-, and chemical-related hepatic diseases. *Clin. Chem.* **56**, 1830–1838.
19. Zhang X., Zhang Z., DaiF., Shi B., Chen L., Zhang X., Zang G., Zhang J., Chen X., Qian F. 2014. Comparison of circulating, hepatocyte specific messenger RNA and microRNA as biomarkers for chronic hepatitis B and C. *PLoS ONE.* **9**, e92112.