Circulating microRNA-122 as a potential biomarker for Hepatitis C Virus-Induced Hepatocellular Carcinoma

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Article

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Abstract

The features of translational repression by micro-RNA can cause various diseases in humans. The liver-specific microRNA (mir122) is primarily involved in tissue tropism during HCV infection. This study focuses on evaluating host serum miR-122 as a prognostic marker in HCV infection. A positive expression of miR-122 was observed in CHC patients followed by HCC patients compared to healthy controls. A difference in median levels of miR-122 expression in CHC and HCC patients ($p < 0.000$) was found in contrast to Cirrhosis patients ($p = 0.511$). The serum miR-122 expression was found threefold higher in liver cirrhosis patients than chronic hepatitis. Further, the AUROC analysis of miR-122 expression profile can efficiently distinguish CHC patients (AUROC = 0.978, p = 0.000, 95% CI = 0.958 to 0.998) and HCC from healthy controls (AUROC = 0.971, p = 0.000, 95% CI = 0.944 to 0.997). Moreover, ROC curve analysis significantly distinguished between CHC patients from Cirrhosis patients (AUROC = 0.955, p = 0.000, 95% CI = 0.925 to 0.986) but not CHC from HCC patients (AUROC = 0.584, p = 0.104, 95% CI = 0.485 to 0.684). This study revealed a substantial correlation of miR-122 with HCV viral load (r = 0.56, p = 0.000), ALT (r = 0.67, p = 0.000) and AST (r = 0.65, p = 0.000) levels. Serum miR-122 can potentially serve as a promising prognostic tool for HCV induced HCC.

Introduction

Hepatitis C Virus (HCV) is an enveloped, positive stranded RNA virus with a genome size of 9.6kb. It belongs to genus *Hepacivirus* in the family *Flaviviridae*. Persistent HCV infection is one of the leading causes of liver disease worldwide and leads to liver cirrhosis and Hepatocellular carcinoma (HCC) [1–3]. HCV genotype 3 is the most common in India and Northeast India has a similar prevalence rate as with the rest of the country [4–6].

MicroRNAs (miRNAs) are small non-coding endogenous RNAs that regulate up to 60% of the expression of cellular mRNAs. miRNAs mostly repress their targets by interaction with the 3' untranslated region (UTR), however, miRNAs may cause upregulation of their targets through interaction in a non-3' UTR region of the mRNA [7].The prospect of using miRNAs as prognostic markers is of interest and has been reported by various studies. MicroRNA changes in disease such as HCC and liver fibrosis, their sensitive detection by quantitative PCR, the non-invasive nature of its assessment make them attractive biomarkers for studying modulation of hepatotrophic virus infection [8]. Also as miRNAs are not targeted by ribonucleases they remain extremely stable in clinical samples [9–11]. miR-122 is a liver specific miRNA whose biological functions are to maintain liver homeostasis, cholesterol metabolism and foetal liver development [7] and is hardly expressed in other tissues [12]. The abundance of miR-122 in the liver helps HCV to promote replication and translation of its genome [2,13,14].

Circulating miR-122 in serum has previously been identified as biomarker for the detection of HCV induced liver injury [9,15–17]. Since the increasing trend of disease burden of HCV can only be tackled with easy and early diagnosis before treatment, the present study aimed to evaluate the diagnostic potential

of serum miR-122 levels in HCV genotype 3 infected patients with chronic infection and cirrhosis from Northeast India.

Materials And Methods

Study subjects

Serum was collected from 192 patients attending the Outpatient Department of Department of Gastroenterology, Gauhati Medical College and Hospital, Guwahati India with informed consent. Patients with Anti-HCV antibodies and HCV RNA positive were included in the study. All patients were confirmed to have HCV genotype 3 infections. Patients with HIV or HBV co-infection, diabetes, alcoholic liver disease, autoimmune or haematological diseases and abnormal liver ultrasounds were excluded from the study.

72 patients were categorised as CHC (Chronic Hepatitis C), while 64 had liver cirrhosis and 56 were diagnosed with HCC. The presence of liver cirrhosis was ascertained by the occurrence of ascites, esophageal varices, splenomegaly, jaundice, imaging and liver biopsies (when available). HCC was diagnosed with 4-phase multi-detector computed tomography (CT) scan, dynamic contrast-enhanced magnetic resonance imaging (MRI) as mentioned elsewhere [18].

Serum from N=40 sex and age matched healthy individuals were taken as controls. All of the controls were selected on the basis of normal liver function tests, normal ultrasound of liver, and absence of viral markers, autoimmune hepatitis and diabetes. Informed consent was obtained from all individual participants included in the study. The study was approved by Institutional ethics committee of Gauhati Medical College and Hospital.

HCV RNA extraction, quantification and genotyping

QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) was used for extracting HCV RNA from serum of HCV infected patients according to manufacturer's protocol and stored at −80 °C. HCV viral load was quantified using Artus HCV RG RT-PCR Kit (Qiagen, Valencia, CA, USA) on Rotor-Gene Q 5plex Platform (Qiagen, Valencia, CA, USA). For HCV genotyping 5'UTR-core region was PCR amplified with specific primers and 173bp product was sequenced to determine the HCV genotype.

miRNA isolation

Total RNA was isolated from whole blood samples using the miRNeasy Minikit (Qiagen, Valencia, CA, USA) followed by miRNA enrichment. Briefly, 100 µl of patient serum was taken with 500 µl of QIAzol lysis reagent and incubated at room temperature for 5 minutes. 140 µl of chloroform was added and vortex to mix for 15 s and incubated at room temperature for 2 minutes. Two separate phases were obtained by centrifuging at 12000 x g at 4□C for 15 minutes. For purification of only miRNA Upper aqueous phase was transferred into new sterile tubes and one volume of 70% ethanol was added and mixed by pipetting. Up to 700 µl of the mixture was added onto RNeasy mini spin column placed in a

clean 2ml collection tube and centrifuged at 8000xg at room temperature for 15s. 450 µl of 100% ethanol was added to the flow through and mixed thoroughly by vortex. RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA, USA) was used for the purification of miRNA fraction from total RNA. 700 µl of the mix was added onto the RNeasy MinElute spin column placed in a 2ml collection tube, and centrifuged at 8000xg at room temperature for 15s. The flow through was discarded and the process repeated for the whole mix volume. 700 µl buffer RWT was added onto the column and centrifuged at 8000xg for 15s. 500 µl of buffer RPE was added onto the column, centrifuged at 8000xg at room temperature for 15 Secs and the flow through discarded. 500 µl of 80% ethanol was added to the column, centrifuged at 8000xg at room temperature for 2 min and flow through was discarded. A new 2ml spin column was used and again centrifuged at 8000xg at room temperature for 5 min. 14 µl of RNase-free water was added onto the column and centrifuged at 8000xg to elute miRNA enriched fraction.

Reverse Transcription

10ng of miRNA was used in total volume of 20 µl to carry out reverse transcription by incubating for 60min at 37°C and 5min at 95°C using miScript PCR Starter Kit (Qiagen, Valencia, CA, USA) according to manufacturer's protocol.

Quantitative real-time PCR

Mature miR-122 expression was carried out by quantitative real time PCR using miScript PCR Starter Kit and miScript SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) with Hs_miR-122a_1 miScript Primer Assay (Qiagen, Valencia, CA, USA) which targets hsa-miR-122-5p. Relative quantification was done using $2^{-\Delta\Delta CT}$ method using U6-snRNA as the internal reference.

Statistical analysis

Statistical calculations were carried out using Statistical Package for the Social Sciences version 16.0 software (SPSS Inc., Chicago, IL) or GraphPad Prism-5.0 (GraphPad Software, CA, USA). A Chi square test or Fischer exact test was performed to compare categorical data. Comparison of independent samples from two groups was performed using the Mann-Whitney U-test. Spearman's nonparametric rank test was used to determine nonparametric statistical significance. The correlation coefficients (r) were calculated using Spearman correlation. Receiver operating characteristic (ROC) analysis was performed to assess the diagnostic and prognostic accuracy and area under the curve (AUC) was calculated. AUROC less than 0.60 were considered unreliable for ROC curve. $p < 0.05$ was considered to be statistically significant.

Results

Demographic and laboratory characteristics of the HCV patients and control group

The clinical and laboratory data of the patients are summarized in Table 1. The average age of Cirrhosis and HCC patients was found higher than both healthy controls and CHC patients ($p = 0.0001$). A significant difference was observed for liver enzyme ALT and AST level between controls and all other groups of patients ($p = 0.0001$). ALT and AST were also significantly elevated in HCC patients than CHC and Cirrhosis patients ($p = 0.0001$). Similarly, difference was observed for total bilirubin ($p = 0.0001$) and serum albumin level ($p = 0.0001$) in cirrhosis and HCC group upon comparison with healthy control. However, no difference was observed while comparing healthy controls with CHC patients. Also, no significant variation was observed between the occurrence of Ascites but Child Pugh score was significantly different among the Cirrhosis and HCC patients ($p = 0.0112$).

Serum levels of miR-122

Relative quantification of serum miR-122 was carried out using U6 snRNA as a reference. The highest expression of miR-122 was found in CHC patients followed by HCC patients with mean fold changes of 5.02 ± 1.48 and 4.46 ± 1.26 respectively in comparison to controls. Patients with Liver Cirrhosis had lesser miR-122 expression as evidenced by a fold change of 2.46 ± 0.84 in comparison to healthy controls. A significant difference in median levels (calculated using Mann-Whitney U-Test) of miR-122 expression was observed in CHC and HCC patients ($p < 0.000$) upon comparison with healthy control in contrast to Cirrhosis patients ($p = 0.511$).

Mann-Whitney U-Test was also performed for serum miR-122 expression levels among different disease states in HCV infected patients. A significant difference was found between CHC and Liver Cirrhosis patients ($p < 0.0001$) but not with HCC patients ($p = 0.0446$). Also, while comparing miR-122 expression for Liver Cirrhosis and HCC patients ($p < 0.0001$) a significant difference was observed. Median levels of miR122 expression across the studied groups were compared by using Kruskal-Wallis test and found to be significantly different ($p < 0.0001$) Fig:1.

ROC curve analysis

The diagnostic ability of miR-122 as a non-invasive diagnostic biomarker for HCC in HCV infected patients was analysed by generating ROC curves. ROC analysis demonstrated that miR-122 expression profile can efficiently distinguish CHC patients (AUROC = 0.978, $p = 0.000$, 95% Confidence Interval = 0.958 to 0.998) and HCC from healthy controls (AUROC = 0.971, $p = 0.000$, 95% Confidence Interval = 0.944 to 0.997). However, miR-122 though had a reduced diagnostic ability to distinguish between Cirrhosis and healthy controls (AUROC = 0.632, $p = 0.024$, 95% Confidence Interval = 0.524 to 0.741). Further, ROC curve analysis also showed efficient discrimination of CHC patients from Cirrhosis patients (AUROC = 0.955, $p = 0.000$, 95% Confidence Interval = 0.925 to 0.986) but not CHC from HCC patients (AUROC = 0.584, $p = 0.104$, 95% Confidence Interval = 0.485 to 0.684). Also serum miR-122 level exhibit an important diagnostic value through its ability to distinguish Cirrhosis patients from HCC patients (AUROC = 0.935, $p = 0.000$, 95% Confidence Interval = 0.895 to 0.975) based on its altered expression profile in different disease state. (Fig 2)

miR-122 expression and its association with HCV viral load

High HCV viral load was not significantly associated with development of cirrhosis or HCC (p = 0.1908, Table No.1). We analysed the association of miR-122 expression with viral load in HCV infected patients. Spearman rank correlation (r) analysis was used to determine association of miR-122 expression with Viral load in (r = 0.56, 95% confidence interval: 0.400 to 0.737, p = 0.000). Viral load was plotted against miR-122 expression as shown in Fig 3a thus establishing association of miR-122 with disease severity in terms of viral load.

miR-122 Fold change with Liver Function Tests in HCC patients

To evaluate whether serum miR-122 levels correlate with serum necro-inflammatory markers ALT, AST, AST/ALT, bilirubin, and albumin in HCC patients, Spearman rank correlation (r) analysis was performed. miR-122 had significant positive correlation with serum ALT (r = 0.544, 95% confidence interval: 0.321 to 0.709, p< 0.0001) and AST (r = 0.532, 95% confidence interval: 0.306 to 0.701, p <0.0001) Fig: 3(a), 3(b), 3(c) and Table: 2.

Discussion

The role of MicroRNAs are ascertain to be vital in virus–host interactions, pathogenesis and host resistance via regulation of post-transcriptional or translational modification. The abundance of hepatic microRNA-122 has made it the most commonly targeted biomarker of liver disease including CHC infection. A non-invasive technique to recognize the severity of liver disease by circulating serum miR-122 assessment essentially can become a preferable choice of diagnosis [2,10,15,19–21].

MiR-122 expression levels of HCV patients are deregulated and distinct from healthy controls, thereby making miR-122 a possible biomarker [14,16,17,22]. Circulating miR-122 has been observed to be significantly high in serum of CHC patients in comparison to healthy controls but most of the studies have not included HCV genotype 3 at a large scale [23]. in a previous study by Cheng et al. 2014 higher miR-122 was reported in PBMCs of HCV patients [24]. Similar results were also reported in study conducted in HCV genotype 3 infected patients from North India, however, the number of patients analysed was low (n = 25) [6]. In contrast, hepatic and blood expression levels of miR-122 was not found to be positively correlated among HCV genotype 3 patients [25]. Indeed, in the present study serum miR-122 was found two-fold higher in CHC patients in compared to normal controls in Northeast Indian population. ROC curve analysis revealed miR-122 in serum of HCV genotype 3 infected patients could be used as a diagnostic tool for HCV infection which is consistent with the previous research findings [26].

The association of hepatic miR-122 levels and extent of liver fibrosis is not yet established. Hepatic miR-122 expression level is reduced significantly with the severity of liver fibrosis in patients with chronic HCV infection [3,27,28] while in another study liver fibrosis stage was not significantly correlated with miR-122 [2,29]. The serum miR-122 has also been previously reported as a potent diagnostic marker in determining

liver injury and distinguishing viral and non-viral etiology. [30,31]. In our study ROC analysis showed that serum miR-122 can be an extremely sensitive diagnostic tool for determining the disease status in CHC patients. MiR-122 has been widely implicated in the diagnosis of HCC, with higher miR-122 levels in comparison to controls [10,11,32]. Recently Motawi et al. have reported non-significant differences in miR-122 expression in HCC patients with that in controls [20]. In case of patients with liver cirrhosis serum miR-122 levels were significantly reduced in comparison to that of CHC patients with almost 3 fold reduction. This is in concurrence with previously reported studies and supports the notion that with hepatocyte destruction and replacement with fibrous tissue, miR-122 export from liver decreases [10].

In several studies on HCV, that have reported no correlation of miR-122 expression in serum with HCV viral load [3,19,24,33]. Hepatic miR-122 expression was also not correlated with serum HCV viral load [34,35] However, few contrasting results have been reported with the association of decreased hepatic miR-122 with increased viral load [27,36]. In HCV genotype 3 infections no correlation was found between both hepatic and serum miR-122 levels with viral load [25]. Recently, Kumar et al reported a strong correlation of miR-122 in serum with viral load in HCV genotype 3 infections [6]. We also observed significant positive correlation of miR-122 with serum HCV genotype 3 RNA levels.

Variations in miR-122 concentrations in serum or plasma have been established as more specific for liver diseases in comparison to serum transaminases, which potentially can originate from non-liver based sources [24]. Serum miR-122 concentrations were correlated with higher AST and ALT levels in Egyptian HCV genotype 4 patients and also in genotype 1 patients [10,11]. However, correlation between serum microRNA-122 and ALT levels was observed only in acute hepatitis patients and not in CHC patients in genotype 1 infection [33]. In another study serum miR-122 and miR-21 were weakly correlated with ALT and AST in Chronic Hepatitis B (CHB) and CHC patients of multiple genotypes [15]. Previous studies focused on HCV genotype 3 have indicated that serum miR-122 levels were significantly higher with strong correlation with elevated ALT, AST and inflammatory scores [2,6]. Our study on HCV genotype 3 patients from Northeast India also followed a similar trend displaying a significant correlation of liver transaminases with serum miR-122 levels.

Conclusion

In conclusion, our study showed that serum miR-122 can be used to diagnose liver injury in CHC patients. No study has been previously reported the predictive ability of serum miR-122 levels in HCV genotype 3 infected patients from Northeast India.

Declarations

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of Interest: The authors declare that they have no conflict of interest.

Availability of Data and Materials: The data generated and/or analysed during the current study are not made publicly available as consent of sharing of raw data is not obtained from all patients, but the same will be available from the corresponding author on reasonable request.

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Tables

Table 1: Clinical characteristics of the patient groups recruited in the study

	Healthy controls (n = 40)	CHC (n=72)	Cirrhosis (n=64)	HCC (n=56)	p value
Age, years	41.9 ± 13.2	39.7 ± 15.1	51.5 ± 9.3[ab]	53.2 ± 12.4[ab]	0.0001
Male Gender, n (%)	30 (75.0)	48 (66.7)	53 (82.8)[b]	43 (76.8)	0.0485
Leucocytes, x 10^3/µL	8.1 ± 2.4	10.7 ± 5.3[a]	6.1 ± 4.1[ab]	6.1 ± 4.6[ab]	0.0062
Haemoglobin, g%	13.2 ± 2.4	10.6 ± 1.2[a]	9.3 ± 2.6[ab]	8.5 ± 3.5[ab]	0.0001
ALT, IU/L	43.2 ± 13.5	70.8 ± 15.6[a]	87.3 ± 24.1[ab]	116.3 ± 18.6[abc]	0.0001
AST, IU/L	37.1 ± 17.8	72.8 ± 20.5[a]	96.6 ± 28.2[ab]	109.7 ± 14.9[abc]	0.0021
ALP, IU/L	-	219.88 ± 102.39	317.62 ± 171.51	317.62 ± 171.51	-
Total Bilirubin, mg/dL	1.8 ± 1.2	2.2 ± 1.7	2.4 ± 1.6[ab]	3.1 ± 1.8[abc]	0.0001
Serum Albumin, g/dL	3.8 ± 1.8	2.9 ± 2.1[a]	2.6 ± 1.2[a]	2.2 ± 1.4[abc]	0.0001
Ascites (Yes/No)	0/40	0/72	46/18	38/18	0.3891
Child score (A/B/C)	-	-	12/19/33	18/24/14	0.0112
Viral Load (<4x 10^5, n (%)/ ≥4x 10^5, n (%))	0	44 (61.1)/28 (38.9)	37 (55.2)/27 (44.8)	29 (51.8)/ 27 (42.2)	0.1902

Data are expressed as mean ± Standard Deviation

[a] denotes significant difference from controls, [b] denotes significant difference from CHC, [c] denotes significant difference from Cirhossis

Table 2: Pearson correlation of serum miR-122 levels in liver cancer patients with routine laboratory parameters

Parameters	Cirrhosis		HCC	
	r	P value	r	P value
ALT, IU/L	0.04	0.74	0.55	< 0.0001
AST, IU/L	0.07	0.53	0.53	< 0.0001
Viral Load	0.14	0.212	0.21	0.120

Figures

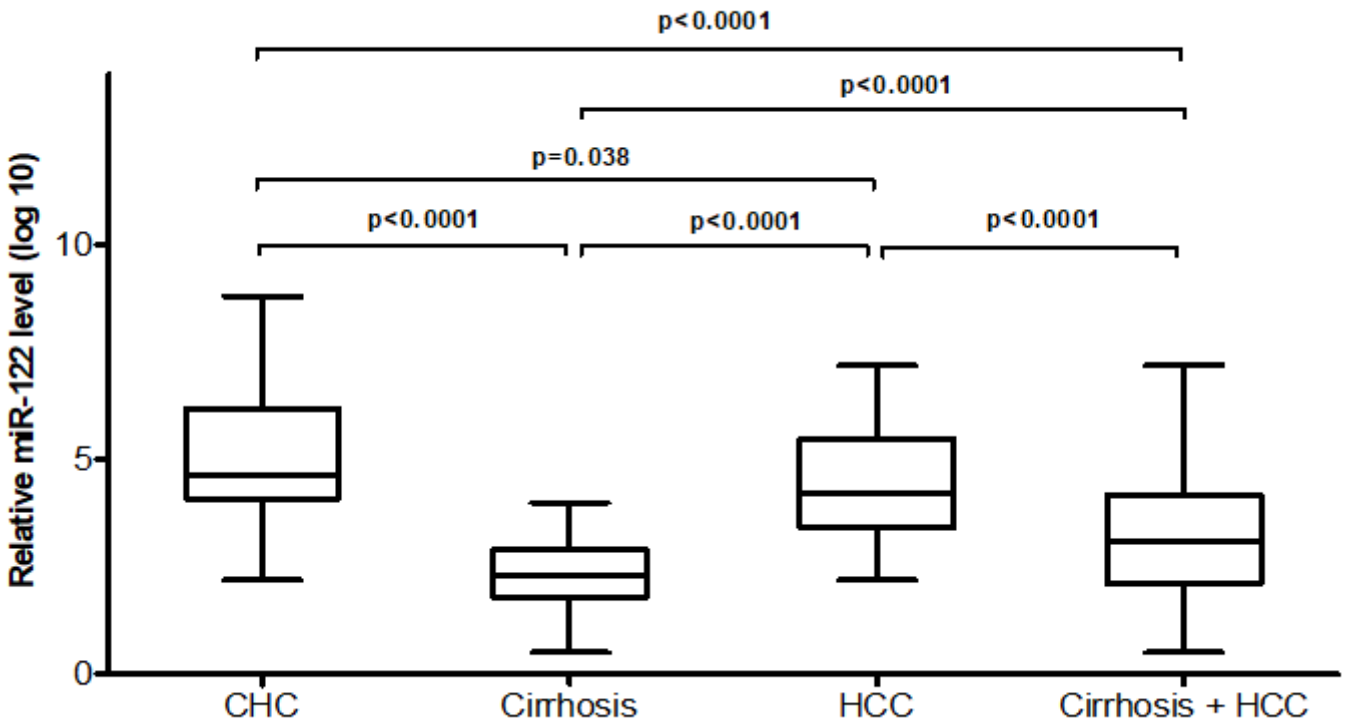


Figure 1

Comparison of relative miR-122 expression among different groups in CHC patients, Cirrhosis and HCC patients. P-value< 0.05 represents statistically significant correlation

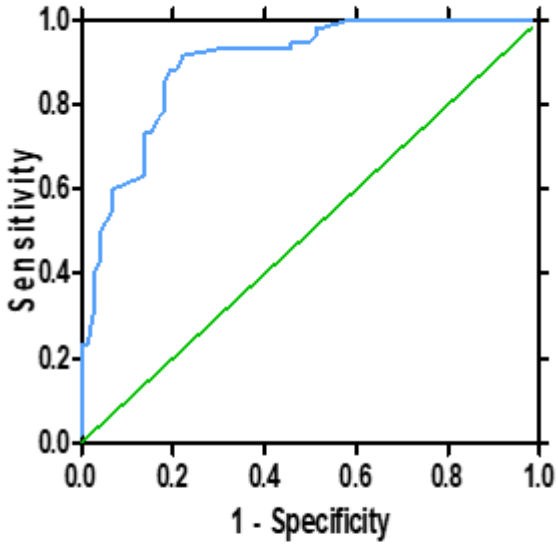
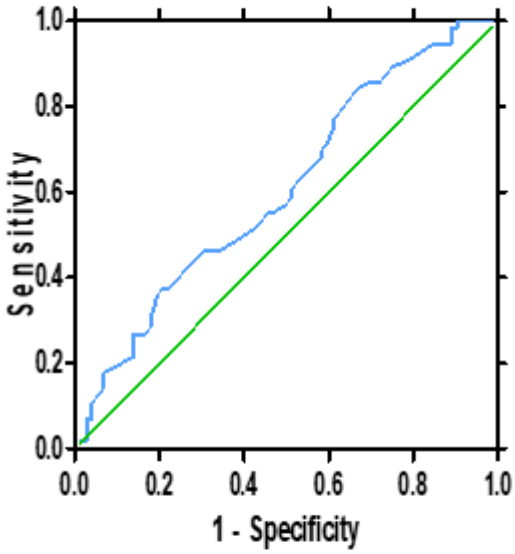
Fig 2 (a): CHC vs Cirrhosis	Fig 2 (b): CHC vs HCC
	
AUROC = 0.8964 p value < 0.0001 95% CI = 0.8440 to 0.9488	AUROC = 0.6066 p value = 0.03894 95% CI = 0.5088 to 0.7045

Figure 2

ROC curve to determine diagnostic ability of miR-122 expression to distinguish, Fig: 2(a) CHC patients from Cirrhosis (AUROC= 0.728, p value= 0.000, 95% Confidence Interval = 0.616 to 0.810). Fig2 (b): CLD cases from cirrhosis (AUROC= 0.931, p value= 0.000, 95% Confidence Interval = 0.868 to 0.994).

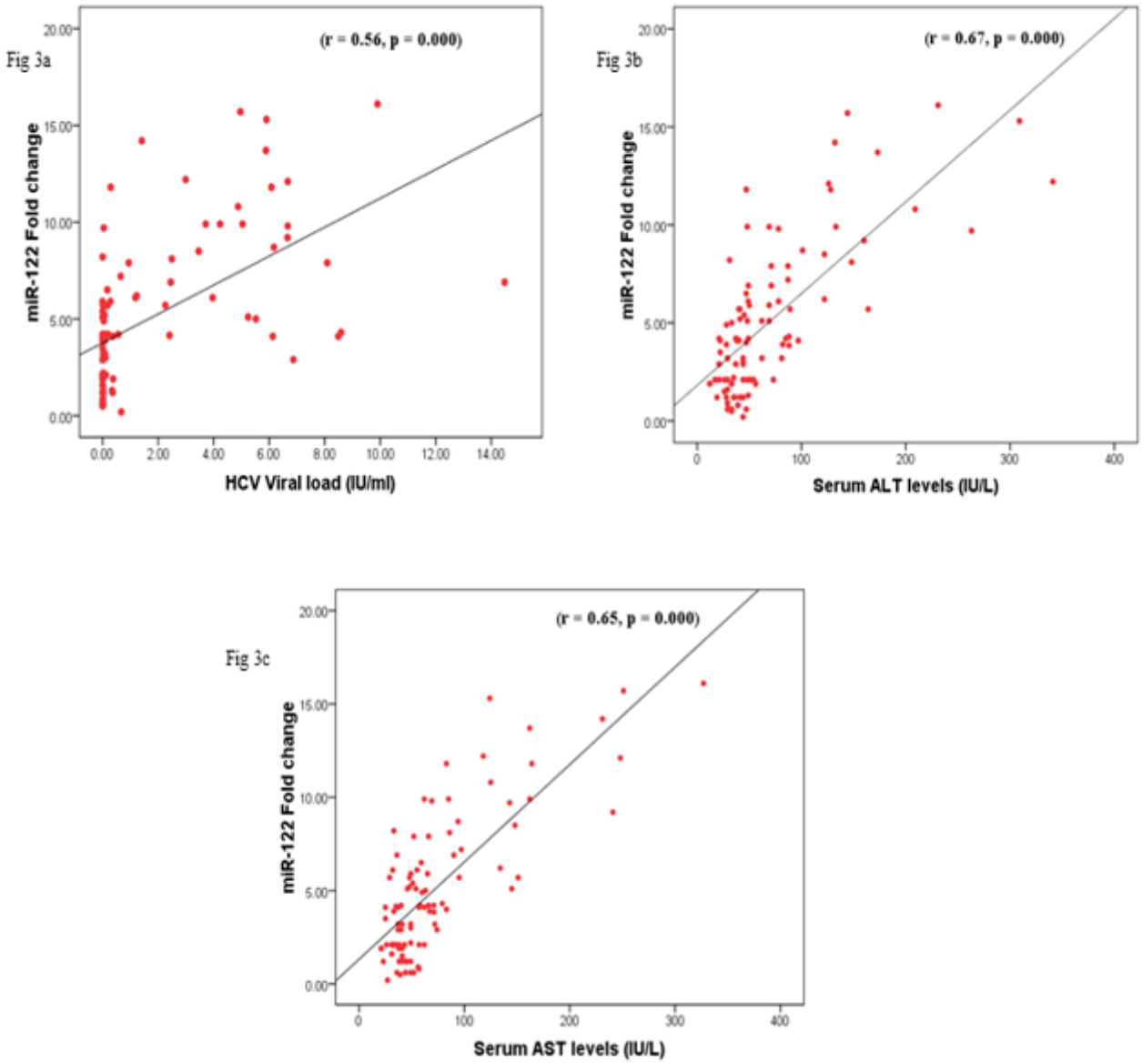


Figure 3

Pearson correlation dot plot representing miR-122 Fold change plot vs HCV viral load (Fig:3a), serum ALT Level(Fig:3b) and Serum AST level (Fig: 3c)