Expression Analysis of MicroRNA-21 and MicroRNA-122 in Hepatocellular Carcinoma

Dipu Bharali [*], Basu D. Banerjee [†], Mausumi Bharadwaj [‡], Syed A. Husain [§], Premashis Kar [*]

[*]Department of Medicine, Maulana Azad Medical College, New Delhi, 02, India, [†]Department of Biochemistry, University College of Medical Sciences, Dilshad Graden, Delhi, 65, India, [‡]Division of Molecular Genetics, National Institute Cancer Prevention and Research, Noida, UP, India and [§]Department of Biosciences, Jamia Millia Islamia University, New Delhi, India

Background/objective: **Hepatocellular carcinoma (HCC) is a multistep process starting from chronic hepatitis (CH) that progress through cirrhosis to HCC. The expression level of microRNA (miRNA) was found to be deregulated in HCC. The study was designed to find out whether the expression level of miR-21 and miR-122 was deregulated in HCC compared to controls without HCC.** ***Methods:*** **Real-time quantitative polymerase chain reaction was performed to find out the miRNA expression level using Ct value followed by statistical analysis where *P* value ≤ 0.05 was considered as significant.** ***Results:*** **Overexpression of miR-21 and miR-122 in HCC was detected. All changes in the expression level of miR-21 and miR-122 were due to HCC compared with healthy control, CH, and liver cirrhosis. Hence miR-21 and miR-122 are suitable to differentiate HCC with an efficient diagnostic power of sensitivity, specificity, and expression level, but they might not have any role in patients' survival.** ***Conclusion:*** **miR-21 and miR-122 could be considered as potential markers of HCC screening molecule in addition to other approved markers. However the current study is limited to expression levels of miRNAs from serum; therefore, it needs further validated study in a large group of population to fulfill all the criteria of a biomarker.** **(J Clin Exp Hepatol 2019;9:294–301)**

Hepatocellular carcinoma (HCC) is the most common cancer worldwide and accounts for about 80% of primary liver cancer tumor in Pacific and Asia regions.[1] HCC is a multistep process starting from chronic hepatitis (CH) that progress through cirrhosis to HCC. Most of the HCC cases are diagnosed at late or advanced stages. Owing to a lack of curative treatment options, HCCs are associated with poor prognosis and low survival rates.[2] MicroRNAs (miRNAs) deregulation in HCC has been studied by earlier published literature. miRNAs are only 17–25 ribonucleotides long, a class of single stranded, non-coding, and evolutionarily conserved RNA sequences. There are several miRNAs those are relevantly associated with HCC. miR-21 and miR-122 were suggested to be the most common cancer-associated miRNAs among several miRNAs by earlier existing literature, and both the miRs are the selective miRNA associated with HCC pathway.[3] The expression profiling of these miRNAs can be an important tool for diagnostics and treatment of disease. Many transcription factors,[4] methylation status of miRNA genes,[5,6] receptors such as nuclear or cellular receptors[7] act as factors to regulate miRNA expression in a tissue-specific and disease state–specific fashion, and some miRNAs are regulated by well-established tumor suppressor or oncogene pathways such as TP53, MYC, and RAS.[4] miRNAs act as either tumor suppressors or oncogenes. Downregulation or loss of miRNAs with tumor suppressor function may increase translation of oncogenes and hence formation of excess oncogenic proteins, leading to tumor formation. In contrast, upregulation of oncogenic miRNAs may block tumor suppressor genes and also lead to tumor formation.[8] miR-21 is considered as oncogenic miRNA, while miR-122 is a tumor suppressor miRNA that regulates intrahepatic metastasis of HCC.[9] The mechanisms of regulation of miRNA expression involve either transcriptional changes in gene expression and promoter hypermethylation or post-transcriptional changes in miRNA processing. At the transcriptional level, expression of miRNA genes can change together with (intragenic miRNAs) their host genes or independently of (intergenic miRNAs) their host genes. Intergenic miRNAs have their own promoters, are expressed independently, and can be regulated by separate transcription factors. On the post-transcriptional level, the

Keywords: HCC, HBV, LC, miR, RT PCR
Received: 22.2.2016; *Accepted:* 7.7.2018; *Available online 17 July 2018*
Address for correspondence: Dr. Premashis Kar Director & HOD, Department of Gastroenterology and Hepatology, Max Super Specialty Hospital Vaishali, Delhi NCR, Pin Code – 201012, India.
E-mail: premashishkar@gmail.com
Abbreviations: Ct: Threshold cycle; HCC: Hepatocellular Carcinoma; LC: Liver Cirrhosis; miRNA: MicroRNA; RT PCR: Real-time Polymerase Chain Reaction
https://doi.org/10.1016/j.jceh.2018.07.005

expression of miRNAs can be downregulated due to changes in the activity of key miRNA biogenesis enzymes such as Dicer and Drosha. The activity of these enzymes can also be affected by mutations or epigenetic modifications.

Deregulation of miRNAs might relate to development of HCC. miRNAs are highly and directly associated with the gene expression regulation as regulator in HCC. miRs also involved in protein translation by regulating target mRNAs. Moreover, growing evidence suggests that miRNAs play an important role in the pathogenesis and development of HCC.[10] Early studies have shown that miRNAs have critical roles in HCC progression by targeting many critical protein-coding genes, thereby contributing to the promotion of cell proliferation; the avoidance of apoptosis, inducing via angiogenesis; and the activation of invasion and metastasis pathways. Although aberrant miRNA expressions have been observed in different types of cancer, and the miRNA downregulated in HCC promotes apoptosis and suppresses tumorigenicity.[11]

Clinical relevance of miRNA-based therapy to build a whole new area of miRNA therapy in human cancer is developed to explore by various research groups and pharmaceutical companies across the globe and therefore consequently few miRNAs have entered the preclinical and clinical stage and are soon expected to be available in the market for use in humans.[12] MRX34, a miRNA-34a mimic, is currently in an ongoing phase I clinical trial, and this therapy has found a manageable safety profile with a partial response observed in one patient. The combination of miRNA-34a with other agents has also proven to exert enhanced antitumor effects. Conversely, many studies have reported that miRNA-34a was upregulated in HCC samples, particularly in those with activation of the beta-catenin pathway. Preclinical studies have shown promising results in the use of a miRNA-34a mimic in HCC as a single agent or as a combination therapy although yet to be fully established.[13]

However, understanding the molecular mechanisms by which miRNAs regulate development and tumorigenesis may lead to novel concepts in the diagnosis and treatment of cancer. The literature pertaining to miRNAs in HCC and the expression level of HCC-associated miRNA is scanty in India, and understanding the underlying reasons for changes in miRNA expression in cancer cells has been paid less attention. Therefore, this study was designed with an aim to detect the differential abnormal expression of miR-21 and miR-122 in HCC patients compared to background CH and liver cirrhosis (LC) without HCC. miR-21 and miR-122 are HCC-associated selective miRNAs.

MATERIALS AND METHODS

Enrollment of Patients, Laboratory Investigation, and Diagnostic Criteria

Methods for Inclusion of Cases and Controls

A total of 50 cases of HCC and 50 controls were enrolled from the medicine ward and Outpatient Department of Medicine and Gastroenterology (OPD) of Lok Nayak Hospital, New Delhi. The sample size was calculated based on "test of two proportion statistical method" and "two means method". Prevalence of HCC cases in OPD and medical ward was one of the criteria for calculation, and number matched controls were included. Age- and gender-matched patients of cases and controls were included in this study.

American Association for the Study of Liver Diseases (AASLD) 2011 evidence was followed to recruit HCC cases, which was based on the AASLD 2011 updated guidelines.[14] The imaging modality used was Triphasic CT abdomen scan which showed the following features: arterial hypervascularization and washout in the portal venous or delayed phase. Alpha-fetoprotein (AFP) ≥200 ng/ml was also an additional criterion followed for diagnosis of HCC.[14]

The control group consisted of CH without LC and HCC (n = 25) and LC without HCC (n = 25) and healthy individuals (n = 10). The diagnosis of CH was based on the recommendation by AASLD 2009 updated guidelines.[15] According to AASLD updated recommendation, the patients were evaluated on the basis of serological analysis for liver function test. The features for inclusion of CH were as follows: (i) persistent or intermittent elevation in alanine aminotransferase (ALT)/aspartate aminotransferase (AST) levels and (ii) liver biopsy showing CH (necroinflammatory score ≥ 4).[15]

The diagnosis of LC was considered on the basis of following criteria:

(i) Presence of ascites, splenomegaly, and shrunken liver; (ii) endoscopic examination showing esophageal varices; and (iii) imaging features for LC such as evidence of surface nodularity (88% sensitive, 82–95% specific), overall coarse and heterogeneous echotexture, and segmental hypertrophy/atrophy.[16]

To ensure the presence of a small HCC in the control group of LC, triphasic CT abdomen scan was performed to differentiate the controls of LC with HCC and without HCC. Different categories of controls such as CH without LC and HCC and with LC without HCC were included in our study to find out the differential expression of miRNA. Healthy controls (HCs) were included as reference control to validate the expression level experiment.

All the cases and controls subjects had given written informed consent for the interview and blood sample collection. The study protocol was approved by the Ethics

Committee of the Maulana Azad Medical College and Associated Lok Nayak Hospitals, New Delhi, and it was conducted in accordance with the declaration of the guidelines of 2011 Helsinki evidences.[17]

A total of 10 ml peripheral blood samples were collected from all the HCC patients and controls without HCC along with healthy donors those are volunteer blood donors with their consent. All the aseptic precautions were taken during handling and subsequent processing of the samples.

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction Assay

Total RNA were isolated from 500 μl serum samples of the cases and controls using commercially available miRVANA PARIS kit for detection of miRNA (Ambion, US) following manufacturer's protocol and were finally resuspended in 45 μl nuclease-free Milli Q (MQ) water. RNA was treated with RNase-free DNase I. 80 ng of total RNA cum miRNA was used as starting material for reverse transcription (RT) to prepare cDNA using RT stem loop primer specific to miR-21 and miR-122 (Invitrogen, USA) and reverse transcription kit (Fermentas, Germany) along with Deoxynucleotides (dNTPS) with Deoxyuridine Triphosphate (dUTP) following manufacturers protocols.

In blood sera from various normal as well as disease condition in various diseases, including chronic hep B and hep C, RnU6b, a SnRNA, were found at constant levels. Therefore, RnU6b SnRNA was used as internal reference control. The U6 small nuclear ribonucleoprotein was used as the inner reference gene for miRs. RnU6b and both miR-21 and miR-122 were quantified by real time RT q-PCR in sera from patients with HCC, LC, and CH without HCC and HCs. miR-21 and miR-122 were subjected to amplification which is based on real-time PCR using rotor gene real-time PCR (Corbett Research, Australia). The expression level of the miR-21 and miR-122 were determined using 40–45 cycles of real-time quantitative PCR assay. Relative expression was calculated using comparative cycle threshold (Ct) values. miRs relative expression was calculated using $2^{-\Delta Ct}$, $\Delta Ct = Ct(miR) - Ct(U6)$. Relative quantification of miRs expression in HCC versus controls without HCC were calculated using the $2^{-\Delta\Delta Ct}$ method, $\Delta\Delta Ct = \Delta Ct$ (cases group) $- \Delta Ct$(control group).

Statistical Analysis

Statistical significance for correlations was determined using Spearman's nonparametric rank test. Differences between two groups were evaluated by Mann–Whitney U test. P values ≤ 0.05 were considered to be significant. Overall survival rates were calculated according to the Kaplan–Meier method and analyzed by the log-rank test. Univariate and multivariate analyses of the prognostic factors were performed with the Cox proportional hazard analyses. $P < 0.05$ was considered statistically significant.

Survival Analysis

Kaplan–Meier survival curve was analyzed from all the data related to the overall survival of HCC patients in both the miRNA high and miRNA low expression group. Patients with HCC were divided into two groups by the median value of the level of miRNA, high-miR group and low-miR group in the overall survival category. The P value of the Kaplan–Meier analysis was calculated with the log-rank test. The overall survival was defined as the time interval from the date of admission into the hospital with the treatment to death or censored on the last follow-up over telephone.

RESULTS

The mean age (±standard deviation) of HCC patients and control without HCC were 56.55 (±9.53) years and 51.33 (±10.65) years, respectively. Out of 50 cases, 45 were HBV-related HCC cases where 91.11% (41/45) of them were HBsAg positive, and 24.44% (11/45) cases were HBeAg positive. ALT and AST were significantly higher in HCC compared to control without HCC. It was depicted that the gender and age groups were found to be nonsignificant with respect to HCC. On the other hand ALT, AST, albumin, and AFP were significant with respect to HCC (Table 1).

The mean Ct values of U6 were 28.7 (27.1–30.2) in HCC, 29.15 (27.5–30.8) in HC, 28.9 (27.2–30.5) in CH, and 29 (27.4–30.6) in LC. Therefore, U6 in the serum of human blood was used as an internal reference control to normalize sampling variations in RT qPCR.

Expression of Serum miR-21 and miR-122 in HCC

miR-21 expression level was significantly higher in HCC compared with controls without HCC. The miR21 expression difference between HCC and HC were significant ($P = 0.001$). Similarly, there was a significant difference of miR-21 expression in HCC and CH ($P \leq 0.05$) and HCC and LC ($P = 0.004$). miR-122 was highly expressed in HCC compared to controls without HCC. The miR-122 expression difference between HCC and HC were significant ($P = 0.001$). There was also a significant difference of miR-122 expression between HCC and CH (P value ≤ 0.05 [=0.001]) and between HCC and LC (P value ≤ 0.05 [=0.002]).

Association of Expression of miR-21 and miR-122 With Clinicopathological Factors of HCC

The expression of miR-21 was significantly higher in HCC with cirrhosis compared to HCC without cirrhosis ($P = 0.0001$) and that of tumor node metastasis (TNM) stage (III-IV) was higher compared with TNM stage (I-II)

Table 1 Distribution of Baseline Clinical Characteristics.

Characteristics	Cases (HCC) n = 50 (%)	Control (without HCC) n = 50 (%)	OR (95% CI)	*P* value
Gender				
Male	38 (75.55)	36 (73.33)	1.00 (Ref)	0.649
Female	12 (24.45)	14 (26.66)	0.812 (0.331–1.98)	
Age range (years)				
≤45	18 (35.55)	17 (42.22)	1.00	0.656
>45	32 (64.45)	33 (57.78)	0.819 (0.341–1.96)	
ALT (IU/dl)				
>35	45 (88.89)	18 (28.89)	1.00	0.041
≤35	5 (11.11)	32 (71.11)	3.162 (1.032–9.687)	
AST (IU/ml)				
>35	42 (82.22)	11 (13.34)	1.00	0.001
≤35	8 (17.8)	39 (86.66)	1.29 (0.619–3.12)	
Total bilirubin (mg/dL)				
≤2	40 (86.66)	9 (8.88)	1.00	0.864
>2	10 (13.34)	41 (91.11)	1.085 (0.414–2.858)	
Albumin (gm%)				
>3.5	28 (53.34)	43 (98)	1.00	0.001
≤3.5	22 (46.66)	7 (2)	1.13 (15.67–108)	
Alpha-fetoprotein (ng/dl)				
≤400	13 (24.44)	44 (96.8)	1.00	0.0001
>400	37 (75.55)	6 (3.2)	5.6 (2.16–14.67)	

HCC, hepatocellular carcinoma; OR, odds ratio; CI, confidence interval.

(*P* = 0.001). The expression of miR-21 was significantly higher in HCC with cirrhosis compared to cirrhosis (*P* = 0.0001). The expression of miR-21 was significantly higher in HCC without LC compared to cirrhosis (*P* = 0.0001) (Table 3).

The expression of miR-122 was significantly higher in HCC with cirrhosis compared to HCC without cirrhosis (*P* = 0.0001) and that of TNM stage (III-IV) was higher compared to TNM stage (I-II) (*P* = 0.0001). The expression of miR-122 was significantly higher in HCC with cirrhosis compared to cirrhosis (*P* = 0.0001). The expression of miR-122 was significantly higher in HCC without LC compared to cirrhosis (*P* = 0.0001) (Table 3).

Correlating HBV viral load in HCC with miRs expression and HBV viral load in LC with miRs expression reveals that HBV was positively correlated with expression level of miR-21 and miR-122. The correlation between miR-21 and miR-122 expression level and other risk factors, such as age, gender, α-fetoprotein levels, tumor size, and tumor number (*P* > 0.05), were found to be nonsignificant (Table 4).

Table 2 Receiver Operating Characteristic Curve Analysis for Predicting Prognostic and Diagnostic Accuracy of MicroRNAs in Hepatocellular Carcinoma.

Factors	miR21	miR122
Sensitivity	74.1%	72.8%
Specificity	75.0%	71.2%
AUC	0.817 ± 0.048	0.743 ± 0.051
Expression level cut-off value	6.6	4.03
Fold change	3.38	2.01

AUC, area under curve.

Diagnostic Power of miR-21 and miR-122 in Differentiation of HCC Compared With Controls

Receiver operating characteristics (ROC) curve analysis was performed on all the data from all HCC cases and controls without HCC. The area under the ROC curve (AUC) for miR-21 was 0.817 ± 0.048 (confidence interval [CI]: 0.723–0.911), with a *P* value of 0.001. The optimal expression level cut off ($2^{-\Delta\Delta Ct}$) for miR-21 (normalized to Rnu6b) to differentiate HCC from that of controls without

Table 3 Association of Expression of MicroRNAs With Clinicopathological Factors of HCC.

Factors	N (50)	miR-21 level ($2^{-\Delta\Delta Ct}$)		miR-122 level ($2^{-\Delta\Delta Ct}$)	
		miR-21	*P* value	miR-122	*P* value
HCC + LC	43	19.26 ± 3.15	0.0001	10.58 ± 2.07	0.0001
HCC minus liver cirrhosis (−ve)	7	10.63 ± 2.78		6.16 ± 1.58	
AFP					
≥400 ng/dl	34	15.7 ± 4.8	0.639	8.17 ± 2.92	0.729
<400 ng/dl	16	16.44 ± 5.34		9.02 ± 2.61	
Tumor number					
1	30	16.08 ± 5.23	0.725	8.51 ± 2.91	0.291
2	18	15.54 ± 4.5		9.41 ± 2.55	
Tumor size					
≤3 cm	12	15.36 ± 4.8	0.668	9.29 ± 2.34	0.753
>3 cm	37	16.08 ± 5.06		8.64 ± 2.95	
TNM grade					
I–II	38	5.9 ± 2.50	0.0001	6.55 ± 3.9	0.0001
III–IV	12	19.25 ± 3.01		11.66 ± 1.34	
CTP score					
HCC + LC	43	19.26 ± 3.15	0.121	10.58 ± 2.07	0.103
Liver cirrhosis (+ve)	25	16.52 ± 4.75		9.3 ± 2.65	
HCC	7	10.63 ± 2.78		6.16 ± 1.58	
LC	25	16.52 ± 4.75		9.3 ± 2.65	

HCC, hepatocellular carcinoma; LC, liver cirrhosis; TNM, tumor node metastasis; AFP, alpha-fetoprotein; CTP, Child-Turcotte-Pugh.

HCC was 6.6 times with a sensitivity of 74.1%, specificity of 75.0%. The mean fold change of miR-21 expression level was 3.38 times in HCC in comparison to controls without HCC (odds ratio [OR] = 4.00, $P = 0.004$) (Table 2).

The area under the ROC curve (AUC) for miR-122 was 0.743 ± 0.051 (CI: 0.643–0.843), with a *P* value of 0.000. The optimal expression level cut off ($2^{-\Delta\Delta Ct}$) for miR-122 (normalized to Rnu6b) to differentiate HCC from that of controls without HCC was 4.03 times with a sensitivity of 72.8%, specificity of 71.2%. The mean fold change of miR-122 expression level was 2.01 times in HCC in comparison to controls without HCC (OR = 1.216, $P \leq 0.05$) (Table 2).

The Correlation Between miR-21 and miR-122 Expression and the Prognosis of HCC Patients: Survival Analysis

Patients with HCC were divided into two groups by the median value of the serum expression level of miRNAs individually. The Kaplan–Meier survival curve represents overall survival (OS) rates between high-miR group and low-miR group. The differences of overall survival rates between high-miR group and low-miR group for miR-21 and miR-122 were non-significant ($P \geq 0.05$). Multivariate analysis of expression level of all the miRNAs revealed that expression of miR-21 and miR-122 in HCC were independent of other variables in HCC.

DISCUSSION

Genome abnormalities or transcriptome changes have been focused in most of the studies, and the relationship between gradual accumulation of molecular alterations and stepwise HCC progression have been established by the earlier studies.[18,19] It has also been indicated in previous findings that systematic changes in chromosomal deletion or global gene expression are unlikely to be involved in the metastatic formation of primary HCC.[20,21] miRNAs were considered as regulator of this global gene expression in human body. miRNAs are deregulated in many kinds of cancers, and it has been found that the deregulation of miRNAs acts as oncogenes or tumor suppressors in HCC onset and progression.[22] Previous reports showed that the deregulation of miRNAs might play important and different roles in HCC development and progression by various kinds of unknown mechanisms.[23–26]

In current study, miR-21 and miR-122 were found to be elevated or expressed highly in blood serum of HCC patients compared with controls without HCCs. Earlier reports stated that miR-21 is elevated in HCC compared to HCs and CH[27–29] and also elevated in HCC tissues

Table 4 Univariate and Multivariate Analysis of Prognostic Factors Associated With Overall Survival Rates in Patients With HCC.

Parameters	Overall survival	
	Univariate *P* value	Multivariate *P* value
Age (≥45 versus <45 years)	0.11	0.077
Gender (male versus female)	0.13	0.089
Log ALT (≥35 versus <35 U/dl)	**0.001**	0.12
Log AST (≥35 versus <35 U/dl)	**0.001**	0.11
Log bilirubin (≤2 versus >2)	**0.001**	0.091
INR (≤2 versus >2)	**0.001**	0.083
Liver cirrhosis (YES/NO)	0.09	0.058
AFP (≥400 ng/dl/<400 Ng/dl)	0.17	0.08
Tumor number (1/2)	0.11	0.076
Tumor size (≤3 cm/>3 cm)	0.09	0.07
TNM grade (I–II/III–IV)	**0.013**	0.059
MicroRNA 21—RnU6b (high versus low)	0.1	0.12
MicroRNA 122—RnU6b (high versus low)	0.093	0.091

TNM, tumor node metastasis; AFP, alpha-fetoprotein; HCC, hepatocellular carcinoma; INR, international normalized ratio after HCC.
Bold values represent $P < 0.05$ was considered statistically significant. The low and high expression of microRNA level was defined according to its cut-off value, which was defined as the median values of the cohort of patients tested.

compared to the corresponding normal tissue.[30,31] Our results were consistent with the results published by Bihrer et.al. 2011 [28] showing that miR-21 do not differ between patients with CH and HCC. Besides, miR-21 was dramatically elevated in HCC tumor cells, with significant reductions of the expressions of several tumor suppressor genes, including PTEN, PDCD4, RECK and TPM1 (PTEN), and MAP2K3, and it has been suggested that this would be the underlying mechanism by which miR-21 is able to directly target MAP2K3 and inhibit its expression during the carcinogenesis of HCC, at both transcriptional and post-translational levels[32,33]

In our study, miR-122 was found to be overexpressed in HCC compared to controls without HCC. A border line significant difference of elevated serum miR-122 in HCC compared to without HCC has been reported in earlier study by Peng et.al,[29] and the significant diagnostic efficiency of serum miR-122 was suggested. It was suggested that the elevation of expression level of miR-122 in the serum of patients may also reflect liver injury[34–36]

The differentiating power of miR-21 and miR-122 in HCC patients and controls without HCC in current study showed that serum miR-21 and miR-122 may be used in the diagnosis of HCC combining together with other FDA-approved tumor markers to improve the sensitivity and specificity. Recent studies revealed the same fact that circulating miR-21 and miR-122 are both potential diagnostic biomarkers and prognostic factors in HCC[36,37]

The association of risk factors such as age, gender, both Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infection, presence of underlying LC, child paugh score, tumor number, tumor size, and TNM tumor stages of HCC with expression of miR-21 and miR-122 showed a prognostic correlation. Our study results showed that the expression of miR-21 and miR-122 was higher in HCC with underlying LC compared to the cases of HCC without LC. This finding indicated that if the miR-21 and miR-122 is found to be higher in patients of cirrhosis, it would indicate the need of screen for HCC. There was no correlation between the expression level of miR-21 and miR-122 with reference to child paugh C, B, and A in our study, but Bihrer et.al. described a significant correlation of serum miR-21 levels with that of child paugh score ($P = 0.00002$).[27]

All the changes in expression level of miR-21 and miR-122 are due to HCC since the difference of expression level of both the miRs among different groups was found to be highly significant (Table 3). So a major concern about hypothesis of this study that all changes of miRs expression level were due to HCC is valid from our results.

Because there was a significant difference of miR-21 and miR-122 expression level in HCC compared to other possible tentative factors of miRNA deregulation such as LC and HCC with LC groups. Therefore, it could be said that the factors such as LC might not be a responsible for differential expression of miR-21 and miR-122 with respect to HCC and therefore differential expression of miRs could be ascribed to HCC only (Table 3).

Moreover correlating HBV viral load in HCC with miR-21 and miR-122 expression and HBV viral load in LC with miR-21 and miR-122 expression reveals that HBV was positively correlated with miR-21 and miR-122 expression. It does not mean that viral load had direct effect on miR-21 and miR-122 expression, but it may have an indirect effect on miRNA expression. HBV viral load may increase the severity of damage in liver and in turn abnormalities in the pathway associated with HCC, and as a result, miR-21 and miR-122 deregulation increases.

Earlier literature explained that differentially expressed miRNAs in the serum of HCV and HCC patients could be used as noninvasive biomarker for segregation of HCV and HCC patients from healthy individuals where miR-122 showed the highest sensitivity and specificity to stratify HCC and HCV compared to normal individuals and HCC compared to HCV.[38] Although the diagnostic value and suitability of circulating miRNAs have been found inconsistent in the literature for the detection of HCC, a meta-analysis demonstrated systematic evaluation of the diagnostic value of circulating miRNAs. Circulating miRNAs were suggested as having a relatively good diagnostic

value in HCC. Multiple miRNAs compared to single miRNA as well as with serum types compared to plasma types were shown a higher accuracy in diagnosis odds ratio which was found in the subgroup analysis.[39] Although expression patterns of miRNAs are different, the high frequency expression miRNAs such as miR-21, miR-199, and miR-122 might be more specific for the diagnosis of HCC.[39] So, multiple miRNAs in serum have a better diagnostic value. In another study, miR-34a and miR-217 expression was found significantly downregulated in HCC tissues ($P < 0.05$), and also the reduced expression of miR-34a and miR-217 was found to be associated with vascular invasion and advanced TNM stage ($P < 0.05$).[40] Also the Kaplan–Meier survival analysis study revealed that reduced expression of miR-34a and miR-217 was associated with poor overall survival compared to patients with high expression of both the miRNAs. The downregulation of miR-34a and miR-217 was associated with HCC progression and may act as tumor suppressor in HCC.[40] The relative expression levels of miR-122 and miR-22 in HBV-related HCC patients were significantly lower compared to benign liver disease and non-HBV–related HCC patients ($P < 0.05$), while miR-122 and miR-22 levels were negatively correlated with tumor size, lymph node metastasis, TNM stage, pathological type, differentiation grade, LC, AFP, and HBV DNA, all of which were independent risk factors ($P < 0.05$).[41] A systematic review and meta-analysis statements described the potential relationship between miRNAs and HBV or HCV-related liver diseases to identify usefulness of serum/plasma/urine miRNAs as noninvasive biomarkers for early detection of HBV- and HCV-induced HCC development as well as for its prognostic evaluation.[42] It described that some serum/plasma miRNAs such as miR-21, miR-122, mi-125a/b, miR-199a/b, miR-221, miR-222, miR-223, and miR-224 might serve as biomarkers for early diagnosis/prognosis of HCC.[42] Therefore, validation of the potential applicability of miRNAs in the diagnosis of HCC is very important, and therefore, more rigorous studies are necessary to confirm the same.

CONCLUSION

The miRNA expression level was found to be deregulated in HCC compared with controls without HCC. Overexpression of miR-21 and miR-122 in HCC was detected. All changes in the expression level of miR-21 and miR-122 were observed in HCC cases compared with controls without HCC. So miR-21 and miR-122 were found suitable to differentiate HCC with an efficient diagnostic power of sensitivity, specificity, and expression level, but they might not have any role in patients′ survival. Therefore, miR-21 and miR-122 might be considered as potential markers of HCC screening molecule in addition to other panel of approved markers. However, the current study is limited to expression levels of miRNAs from serum; therefore, it needs further study validation in a large group of population to fulfill all the criteria of a biomarker. Our study warrants further study pertaining to miRNA expression levels.

CONFLICTS OF INTEREST

The authors have none to declare.

ACKNOWLEDGMENT

The authors are grateful to the University Grant Commission (UGC) to carry out this research work in the PCR Hepatitis Laboratory, Department of Medicine, MAMC, New Delhi.

REFERENCES

1. Chamberlain J, Sarfati D, Cunningham R, Koea J, Gurney J, Blakely T. Incidence and management of hepatocellular carcinoma among Māori and non-Māori New Zealanders. *Aust N Z J Public Health*. 2013;37(6):520–526.
2. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *J Proc Natl Acad Sci USA*. 2006;103:2257–2261.
3. Farazi Thalia, Hoell Jessica, Morozov Pavel, Tuschl Thomas. microRNAs in human cancer. *J Adv Exp Med Biol*. 2013;774:1–20.
4. Krol J, Loedige I, Filipowicz W. The wide-spread regulation of microRNA biogenesis, function and decay. *J Nat Rev Genet*. 2010; 11:597–610.
5. Han L, Witmer PD, Casey E, Valle D, Sukumar S. DNA methylation regulates MicroRNA expression. *J Cancer Biol Ther*. 2007;6(8): 1284–1288.
6. Saito Y, Jones PA. Epigenetic activation of tumor suppressor microRNAs in human cancer cells. *J Cell Cycle*. 2006;5:2220–2222.
7. Yang Zhihong, Wang Li. Regulation of microRNA expression and function by nuclear receptor signaling. *J Cell Biosci*. 2011;1(1):31.
8. Zhang Baohong, Pan Xiaoping, Cobb George P, Anderson Todd A. microRNAs as oncogenes and tumor suppressors. *J Dev Biol*. 2007;302(1):1–12.
9. Tsai WC, Hsu PW, Lai TC, et al. MicroRNA-122, a tumor suppressor microRNA that regulates intrahepatic metastasis of hepatocellular carcinoma. *Hepatology*. 2009;49(5):1571–1582.
10. Giordano S, Columbano A. MicroRNAs: new tools for diagnosis, prognosis and therapy in HCC? *J Hepatol*. 2013;57(2):840–847.
11. Su H, Yang JR, Xu T, et al. MicroRNA-101, downregulated in hepatocellular carcinoma, promotes apoptosis and suppresses tumorigenicity. *J Cancer Res*. 2009;69:1135–1142.
12. Christopher AF, Kaur RP, Kaur G, Kaur A, Gupta V, Bansal P. MicroRNA therapeutics: Discovering novel targets and developing specific therapy. *J Perspect Clin Res*. 2016 Apr-Jun;7(2):68–74.
13. Saito Y, Nakaoka T, Saito H. MicroRNA-34a as a therapeutic agent against human cancer. *J Clin Med*. 2015;4(11):1951–1959.
14. Bruix Jordi, Sherman Morris. Management of hepatocellular carcinoma: an update, AASLD practice guidelines. *J Hepatol*. 2011;53(3).
15. Lok ASF, McMahon Brian J. *Chronic Hepatitis B: Update*. 2009.
16. Runyon Bruce A. Introduction to the revised American Association for the Study of Liver Diseases practice guideline management of adult patients with ascites due to cirrhosis 2012. AASLD practice guidelines. *Hepatology*. 2013;57(4):1652–1682.
17. WMA declaration of helsinki – Ethical principles for medical research involving human subjects. www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/. Adopted by the 18th WMA General

Assembly, Helsinki, Finland, June 1964 and amended by the: 64th WMA General Assembly, Fortaleza, Brazil, October 2013.
18. Um TH, Kim H, Oh BK, et al. Aberrant CpG island hypermethylation in dysplastic nodules and early HCC of hepatitis B virus-related human multistep hepatocarcinogenesis. *J Hepatol*. 2011;54:939–947.
19. Midorikawa Y, Yamamoto S, Tsuji S, et al. Allelic imbalances and homozygous deletion on 8p23.2 for stepwise progression of hepatocarcinogenesis. *J Hepatol*. 2009;49:513–522.
20. Lee JM, Wong CM, Ng IO. Hepatitis B virus-associated multistep hepatocarcinogenesis: a stepwise increase in allelic alterations. *J Cancer Res*. 2008;68:5988–5996.
21. Ye QH, Qin LX, Forgues M, et al. Predicting hepatitis B virus-positive metastatic hepatocellular carcinomas using gene expression profiling and supervised machine learning. *J Nat Med*. 2003;9:416–423.
22. Wen Jessica, Friedman Joshua R. miR-122 regulates hepatic lipid metabolism and tumor suppression. *J Clin Invest*. 2012;122(8):2773–2776.
23. Zhang J, Yang Y, Yang T, et al. MicroRNA-22, downregulated in hepatocellular carcinoma and correlated with prognosis, suppresses cell proliferation and tumourigenicity. *Brit J Cancer*. 2010;103(8):1215–1220.
24. Jiang J, Gusev Y, Aderca I, et al. Association of microRNA expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. *J Clin Cancer Res*. 2008;14(2):419–427.
25. Ura S, Honda M, Yamashita T, et al. Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma. *J Hepatol*. 2009;49(4):1098–1112.
26. Murakami Y, Yasuda T, Saigo K, et al. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *J Oncogen*. 2006;25(17):2537–2545.
27. Xu J, Wu C, Che X, et al. Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. *J Mol Carcinog*. 2011;50:136–142.
28. Bihrer V, Waidmann O, Friedrich-Rust M, et al. Serum MicroRNA-21 as marker for necroinflammation in hepatitis C patients with and without hepatocellular carcinoma. *J PLoS One*. 2011;6(10)e26971.
29. Qi Peng, Cheng Shu-qun, Wang Hao, Li Nan, Chen Yue-feng, Gao Chun-fang. Serum MicroRNAs as biomarkers for hepatocellular carcinoma in Chinese patients with chronic hepatitis B virus infection. *J PLoS One*. 2011;6(12)e28486.
30. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A*. 2008;105:10513–10518.
31. Iliopoulos D, Jaeger SA, Hirsch HA, Bulyk ML, Struhl K. STAT3 activation of miR-21 and miR-181b-1 via PTEN and CYLD are part of the epigenetic switch linking inflammation to cancer. *J Mol Cell*. 2010;39:493–506.
32. Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA- 21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *J Gastroenterology*. 2007;133(2):647–658.
33. Zhang BG, Li JF, Yu BQ, Zhu ZG, Liu BY, Yan M. microRNA-21 promotes tumor proliferation and invasion in gastric cancer by targeting PTEN. *J Oncol Rep*. 2012;27(4):1019–1026.
34. Lagos-Quintana M, Rauhut R, Yalcin A, et al. Identification of tissue-specific microRNAs from mouse. *Curr Biol*. 2002;12:735–739.
35. Wang K, Zhang S, Marzolf B, et al. Circulating microRNAs, potential biomarkers for drug-induced liver injury. *Proc Natl Acad Sci U S A*. 2009;106:4402–4407.
36. Li JB, Shi WX, Gao YT, et al. Analysis of microRNA expression profiles in human hepatitis B virus-related hepatocellular carcinoma. *Clin Lab*. 2013;59(9–10):1009–1015.
37. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. *J Biol Chem*. 2008;283:1026–1033.
38. Ali HEA, Abdel HR, Effat H, et al. Circulating microRNAs panel as a diagnostic tool for discrimination of HCV-associated hepatocellular carcinoma. *J Clin Res Hepatol Gastroenterol*. 2017;41(4):e51–e62.
39. Ding Y, Yan JI, Fang AN, Zhou WF, Huang L. Circulating miRNAs as novel diagnostic biomarkers in hepatocellular carcinoma detection: a meta-analysis based on 24 articles. *J Oncotarget*. 2017;8(39):66402–66413.
40. Tian YW, Shen Q, Jiang QF, Wang YX, Li K, Xue HZ. Decreased levels of miR-34a and miR-217 act as predictor biomarkers of aggressive progression and poor prognosis in hepatocellular carcinoma. *J Minerva Med*. 2017;108(2):108–113.
41. Qiao DD, Yang J, Lei XF, et al. Expression of microRNA-122 and microRNA-22 in HBV-related liver cancer and the correlation with clinical features. *Eur Rev Med Pharmacol Sci J*. 2017;21(4):742–747.
42. Fiorino S, Bacchi-Reggiani ML, Visani M, et al. MicroRNAs as possible biomarkers for diagnosis and prognosis of hepatitis B- and C-related-hepatocellular-carcinoma. *World J Gastroenterol*. 2016;22(15):3907–3936.