Expression Analysis of Serum microRNA-34a and microRNA-183 in Hepatocellular Carcinoma

Dipu Bharali1*, Hakim B Jebur2, Debabrata Baishya3, Suresh Kumar4, Manash P Sarma1, Mirza Masroor5, Juheb Akhter6, Syed A Husain6, Premashis Kar1

Abstract

Background/objective: HCC is a multistep process starting from chronic hepatitis that progress through cirrhosis to HCC. MicroRNA expression level was found to be deregulated in HCC. To find out whether the expression level of miR-34a and miR-183 was deregulated in HCC compared to controls without HCC. Methods: Real time quantitative PCR was done to find out the miRNA expression level in terms of Ct value followed by statistical analysis. Results: Over-expression of miR-183 and under-expression of miR-34a in HCC was detected. All changes in expression level of miR-34a and miR-183 were found to be due to HCC compared to controls without HCC. So both miR-34a and miR-183 were suitable to differentiate HCC from Cirrhosis and chronic hepatitis with an efficient diagnostic power of sensitivity, specificity and expression level. But they might not have any role in patients’ survival. Conclusion: miR-34a and miR-183 might be considered as potential markers of HCC screening molecule in addition to other approved panel of marker. Our study warrants further expression level study.

Keywords: HCC- hepatocellular carcinoma- LC- liver cirrhosis- miR- micro RNA- RT PCR- Ct -threshold cycle

Introduction

Hepatocellular carcinoma (HCC) is the most common cancers worldwide and accounts for about 80% of primary liver cancer tumor in Pacific and Asia regions (Chamberlain et al., 2013). HCC is a multistep process starting from chronic hepatitis that progress through cirrhosis to HCC. Most of the HCC cases are diagnosed at late or advanced stages and due to a lack of curative treatment options. HCCs are associated with poor prognosis and low survival rates (Volina et al., 2006). MicroRNAs (miRNAs) are deregulated 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 micro RNAs those are relevantly associated with HCC. miR-34a and miR-183 are most common cancer-associated miRNAs among several microRNAs suggested by earlier literature and are the selective micro RNA associated with HCC pathway (Thalia et al., 2013). The expression profiling of these miRNAs can be an important tool for diagnostics and treatment of disease. There are several factors such as transcription and translational factors, methylation status of miRNA genes (Tsai et al., 2009; Krol et al., 2010), receptors such as nuclear or cellular (Chang et al., 2007) 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 (Baoaong et al., 2007). MicroRNAs act as either tumour suppressors or oncogenes. Downregulation or loss of miRNAs with tumour suppressor function may increase translation of oncogenes and hence formation of excess oncogenic proteins, leading to tumour formation. In contrast, upregulation of oncogenic miRNAs may block tumour suppressor genes and also lead to tumour formation (He et al., 2007). miR-34a and miR-183 are considered as oncogenic micro RNA that regulates intrahepatic metastasis of hepatocellular carcinoma (Krol et al., 2010). To understand the molecular mechanisms of miRNA expression changes involved in hepatocellular carcinoma, it is necessary to understand the alterations in miRNA transcriptional regulation. The miRNA and its transcriptional regulators can participate in complex feedback regulation loops such as TP53 regulated mir-34a (He et al., 2007; Han et al., 2007). MiRNA dysregulation has also been linked to changes in epigenetic regulation, such as the methylation status of miRNA genes, which

1Department of Medicine, 2Department of Biochemistry, Maulana Azad Medical College, 3Department of Medical Toxicology, Jamia Hamdard, Delhi, India, 4Department of Pharmacology, Faculty of Medicine, Wasit University, Iraq, 5Department of Bioengineering and Technology, Gauhati University, Assam. *For Correspondence: dipubharali33@gmail.com
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 microRNA 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. Different mutations can alter the expression of microRNAs or promoter methylation can regulate the expression in both cases. On the post-transcriptional level the expression of microRNAs can be downregulated due to changes in the activity of key miRNA biogenesis enzymes, such as Dicer and Drosha. Activity of these enzymes can also be affected by mutations or epigenetic modifications. Activation of nuclear receptors can induce expression of both intergenic and intragenic microRNAs.

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 (Zhihong et al., 2011). 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 microRNA (miRNA) expressions have been observed in different types of cancer and microRNA downregulated in hepatocellular carcinoma promotes apoptosis and suppresses tumorigenicity (Krol et al., 2010).

Clinical relevance of miRNA based therapy to build a whole new area of microRNA therapy in human cancer is developed to explore by various research groups and pharmaceutical companies across the globe and so consequently few miRNAs have entered the preclinical and clinical stage and soon expected to be available in the market for use in humans (Davis et al., 2010). MRX34, a miRNA-34a mimic, is currently in an ongoing phase 1 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 anti-tumour effects. Conversely, many studies have reported that miRNA-34a was up-regulated in HCC samples, particularly in those with activation of the beta-catenin pathway. Pre-clinical 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 (Brevig et al., 2010).

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 microRNAs in HCC and the expression level of HCC associated micro RNA is scanty in India and understanding the underlying reasons for changes in miRNA expression in cancer cells has paid less attention. Therefore this study was designed with an aim to detect the differential abnormal expression of miR-34a and miR-183 in HCC patients compared to background chronic hepatitis and liver cirrhosis without HCC. miR-34a and miR-183 are HCC associated selective micro RNAs.

Materials and Methods

Enrolment of patients, laboratory investigation, 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. Sample size was calculated based on “Test of two proportion statistical method” and “Two means method” with 80% power of the study. Prevalence of HCC cases in OPD and medical ward was one of the criteria for calculation of cases of HCC. Number matched controls were included in our study. Age and gender matched patients of cases and controls were included in this study.

AASLD 2011 evidence were followed to recruit HCC cases which was based on the imaging modalities of Triphasic CT abdomen scan which showed the Arterial hypervascularization, Washout in the portal venous or delayed phase (Bruix et al., 2011). AFP ≥ 200 ng/ml was also an additional criterion followed for diagnosis of HCC.

The control group consisted of chronic hepatitis without liver cirrhosis and HCC (n=25) and liver cirrhosis without HCC (n=25) and healthy individuals (n=10). The diagnosis of chronic hepatitis was based on the recommendation by AASLD 2009 updated guidelines (Anna and Brian, 2009). According to AASLD recommendation the patients was evaluated on the basis serological analysis for liver function test. The factors for inclusion of chronic hepatitis were as follows: i) Persistent or intermittent elevation in ALT/AST levels, ii) Liver biopsy showing chronic hepatitis (necroinflammatory score ≥ 4).

The diagnosis of liver cirrhosis was considered on the basis of following criteria:

i) Presence of Ascites, Splenomegaly, Shrunken liver, ii) Endoscopic examination showing oesophageal varices, v) Imaging features for liver cirrhosis are as follows:

Evidence of Surface nodularity: (88% sensitive, 82-95% specific), Overall coarse and heterogeneous Echotexture, segmental hypertrophy/atrophy (Bruce et al., 2013).

To ensure the presence of a small HCC in the controls with liver cirrhosis Triphasic CT abdomen scan were performed which showed the following features: Arterial hypervascularization, Washout in the portal venous or delayed phase (Bruix et al., 2011).

To find out the differential expression of micro RNA in HCC compared to controls of chronic hepatitis and liver cirrhosis without HCC. Healthy controls were included as reference control to validate the expression results in alterations in their expression levels (Saito et al., 2006; Zhihong et al., 2011).
level experiment.
All the cases and controls subjects had given written informed consent for the interview and blood sample collection. The study was approved by the local ethics committee of MAMC New Delhi and it was conducted in accordance with the declaration of the guidelines of 2011 Helsinki evidences (WMA, 2011)
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 PCR assay
Total RNA were isolated from 500 µl serum sample of the Disease group and Control group using commercially available miRVANA PARIS kit for detection of micro RNA (Ambion, USA) following manufacturer’s protocol and finally resuspended it in 45 µl nuclease free 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-34a and miR-183 (Invitrogen, USA) and reverse transcription kit (Fermentas, Germany) along with dNTPS with 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 Sn RNA 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. Both RnU6b and micro RNA miR34a and miR-183 were quantified by real time RT q-PCR in sera from patients with HCC, liver cirrhosis (LC) and chronic hepatitis (CH) without HCC and healthy controls (HC). miR-34a and miR-183 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-34a and miR-183 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-ΔΔCt, ΔΔCt = Cт (miR) - Cт (U6). Relative quantification of miRs expression in HCC versus controls without HCC were calculated using the 2-ΔΔCt method, ΔΔCt = ΔCт (Cases group) - ΔCт (control group).

Statistical Analysis
Statistical significance for correlations was determined using Spearman’s nonparametric rank test. Differences between two groups were evaluated by Maan-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 were analyzed from all the data related to overall survival of HCC patients in both the micro RNA high and micro RNA 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 Kaplan-Meier estimator was used to evaluate the median survival time of the OS that was based on miRNA expression signature or clinical parameters. Kaplan-Meier survival analysis was done to compare two survival curve between two groups using the log-rank test. 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 (±SD) of HCC patients and control without HCC were 56.55 (± 9.53) years and 51.33 (±10.65) years, respectively. HBsAg were found to be positive in 91.1% of HCC cases and 24.44% cases were HBeAg positive. Out 50 cases 45 were HBV related HCC cases and 91.1% (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 non significant with respect to HCC. On the other hand ALT, AST, Albumin and AFP were significant with respect to HCC.

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

Expression of serum miR-34a and miR-183 in HCC
miR-34a expression level was significantly lower in HCC compared to controls without HCC. The difference of expression of miR-34a between HCC and healthy control (p=.001), between HCC and chronic hepatitis (p-value = .001) and between HCC and liver cirrhosis (p =0.004) were found to be significant.

miR-183 expression level was significantly higher in HCC compared to controls without HCC. The difference of expression of miR-183 between HCC and healthy control (p ≤ 0.05, p=0.001) were significant. There was a significant difference of miR-183 expression between HCC and chronic hepatitis (p value ≤ 0.05 (≈ 0.001)) and between HCC and liver cirrhosis were found to be significant.

Association of expression of miR-34a and miR-183 with clinicopathological factors of HCC
The expression of miR-34a was significantly lower in HCC with cirrhosis compared to HCC without cirrhosis (p-value = 0.0003) and that of TNM stage (III-IV) was lower compared to TNM stage (I-II) (p-value = 0.0001). The expression of miR-34a was significantly lower in HCC with cirrhosis compared to liver cirrhosis (LC)
The expression of miR-34a was significantly lower in HCC without liver cirrhosis compared to liver cirrhosis (LC) (p-value = 0.0001). The expression of miR-183 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 = 0.0016). The expression of miR-183 was significantly higher in HCC without liver cirrhosis compared to liver cirrhosis (LC) (p-value = 0.0001).

Correlating HBV viral load in HCC with miRs expression and HBV viral load in liver cirrhosis with miRs expression reveals that HBV was positively correlated with expression level of miR-183 and miR-34a. The correlation between miR-183 and miR-34a 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 non-significant.

Diagnostic power of miR-34a and miR-183

The area under the ROC curve (AUC) for miR-34a was 0.843 ± 0.036 (CI: 0.823–0.964) (Figure 2C), with a P value of 0.001. The optimal expression level cut off (2−∆∆Ct) for miR-183 (normalized to Rnu6b) to differentiate HCC from that of controls without HCC patients was 9.99 times with a sensitivity of 87.1%, specificity of 88.12%. The mean fold change of miR-183 expression level was 1.23 times in HCC in comparison to controls without HCC (O.R=2.00, p≤0.05) (Table 2).

Patients with HCC were divided into two groups by the median value of the serum expression level of micro RNAs 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-34a and miR183 were non-significant (p ≥ 0.05). Multivariate analysis of expression level of all the microRNAs revealed that expression of miR-34a and miR-183 in HCC were independent of other variables.
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 (Um et al., 2011; Midorikawa et al., 2009). 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 (Lee et al., 2008; Ye et al., 2003). Micro RNAs 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 (Baohong et al., 2007). 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 (Zhang et al., 2010; Jiang et al., 2008)

In current study miR-183 were found to be elevated in blood serum of HCC patients compared to controls without HCCs. It was suggested in existing literatures that the serum levels of circulating microRNAs reflect smaller differences between HCC and either chronic hepatitis or liver cirrhosis and also suggested that the serum exosomal microRNAs might be used as novel serological biomarkers for HCC rather than serum circulating microRNAs (Ura et al., 2009).

miR-183 was also found to be highly expressed in our study. The deregulated miR-183 may behave as oncogene by inhibition of apoptosis in HCC due to repression of the PDCD4 protein expression (Murakami et al., 2006) and also reducing the expression of tumor suppressor
gene AKAP12 in HCC (Sohn et al., 2015). Upregulation of miR-183 in HCC has been suggested by several studies (Li et al., 2010; Goepfert et al., 2010). miR-183 expression was also shown to be significantly higher in HCC compared to controls without HCCs (Li et al., 2013). Over-expression of miR-183/96/182 might be considered as an independent prognostic predictor for HCC patients due to conferring an oncogenic function in HCC cell dissemination (Liang et al., 2013). The diagnostic efficacy of miR-183 in discriminating HCC from chronic hepatitis and liver cirrhosis might be suggested its role in diagnosis of chronic liver diseases. miR-183 upregulation might be associated with onset and progression of HCC has been reported, but not associated with the patient survival.

Our study found miR-34a expression level lower in HCC in comparison to controls without HCC. MiR34a expression was dramatically downregulated or silenced in various cancers including HCC has also been documented (Leung et al., 2015). Aberrant expression of miR-34a has been reported to be involved in the tumorigenesis and progression of various classes of malignancies (Dang et al., 2013). A significant down-regulation of miR34a expression level in HCC cell lines by qRT-PCR has also been demonstrated (Arroyo et al., 2011). Real time RTqPCR data by Li et al., (2009) reported that down-regulation of miR-34a expression was highly significant in HCC compared with controls. Dang et al.,2013 also reported the same results using RTqPCR and confirmed the lower miR-34a expression level in a larger size of population of HCC cases compared to the non-cancerous controls. The underexpression of miR-34a in HCC might indicate the critical role in the hepatocarcinogenesis, as a tumor suppressor miRNA.

The differentiating power of miR-183 and miR-34a in HCC patients and controls without HCC in current study showed that serum miR-183 and miR-34a 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-183 and miR-34a are potential diagnostic biomarkers and prognostic factors in HCC (Liang et al., 2013; Leung et al., 2015; Dang et al., 2013; Arroyo et al., 2011).

The association of correlation of expression level of miR-183 and miR-34a in HCC patients with risk factors like Age, Gender, both HBV and HCV infection, presence of underlying liver cirrhosis, child paugh score, tumor number, tumor size, TNM tumor stages of HCC cases had a prognostic correlation. Our study results showed that the expression of miR-183 was higher in HCC with underlying liver cirrhosis compared to the cases of HCC without liver cirrhosis. This finding indicated that if miR-183 is found to be higher in patients of cirrhosis, it would indicate the need of screen for HCC. We did not find any correlation between the expression level of miR-183 with reference to child paugh C, B, and A in HCC.

A significant difference was found to be seen between the expression level of miR-34a in HCC with child paugh C and HCC with child paugh B and A. Dang et al., (2013) investigated the clinical significance of miR-34a and in vitro contribution of miR-34a on biological functions of human HCCs suggested a supportive result reporting a significant decrease in miR-34a level in HCCs compared to adjacent liver tissues detected by RT-qPCR.

All the changes in expression level of all the miR-34a and miR-183 are due to HCC since the difference of expression level of miR-34a and miR-183 among different groups was found to be highly significant. So major concern about hypothesis of this study that all changes of miRs expression level were due to HCC is valid from the results.

Since there was a significant difference of miR expression level in HCC compared to other possible tentative factors of micro RNA deregulation such as liver cirrhosis and HCC with liver cirrhosis groups. Therefore it could be said that the factors such as liver cirrhosis might not be a responsible for differential expression of miR-34a and miR-183 with respect to HCC and so differential expression of miR-34a and miR-183 could be ascribed to HCC only.

Moreover correlating HBV viral load in HCC with miR expression and HBV viral load in liver cirrhosis with miR expression reveals that HBV was positively correlated with miRs expression. It does not mean that viral load had direct effect on miRNA 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 as a result micro RNA deregulation increases or decrease.

Differentially expressed microRNAs in the serum of HCV and HCC patients could be used as non invasive biomarker for segregation of HCV and HCC patients from healthy subjects was demonstrated with highest sensitivity and specificity to stratify HCC and HCV compared to normal individuals and HCC compared to HCV (Ali et al., 2017). Although the diagnostic value and suitability of circulating miRNAs for the detection of hepatocellular carcinoma have been inconsistent in the literature, 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 hepatocellular carcinoma. 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. Although expression patterns of miRNAs are different, the high frequency expression miRNAs might be more specific for the diagnosis of hepatocellular carcinoma (Ali et al., 2017).

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) (Ding et al., 2017). Kaplan-Meier analysis in te same 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. miR-34a and miR-217 down-regulation was associated with HCC progression and may act as tumor suppressor in HCC (Ding et al., 2017). The expression levels of miRNAs
were found to be negatively correlated with tumor size, lymph node metastasis, TNM stage, pathological type, differentiation grade, liver cirrhosis, AFP and HBV DNA, all of which were independent risk factors (p < 0.05) (Tian et al., 2017). A systematic review and meta-analysis statements were described the potential relationship between miRNAs and HBV or HCV related liver diseases to identify usefulness of serum/plasma/urine miRNAs as non invasive biomarkers for early detection of HBV and HCV induced hepatocellular carcinoma (HCC) development as well as for its prognostic evaluation (Qiao et al., 2017). It described that serum or plasma miRNAs could be serve as biomarkers for early diagnosis or prognosis of HCC (Qiao et al., 2017). Therefore validation of the potential applicability of miRNAs in the diagnosis of hepatocellular carcinoma is very important and so more rigorous studies are necessary to confirm the same.

In conclusion, microRNA expression level were found to be deregulated in HCC compared to controls without HCC. Over-expression of miR-183 and under-expression of miR-34a in HCC compared to controls without HCC was detected. All changes in expression level of all the miR-34a and miR-183 was observed due to HCC compared to controls without HCC such as liver cirrhosis. So miR-34a and miR-183 were suitable to differentiate HCC with an efficient diagnostic power of sensitivity, specificity and expression level. Therefore these might be considered as potential markers of HCC screening panel of molecule in addition to other approved marker.

Abbreviations

HCC- Hepatocellular Carcinoma, LC- Liver Cirrhosis, miR- micro RNA, RT PCR- Real time Polymerase Chain Reaction, Ct -Threshold cycle.

Conflict of Interest
None to be declared.

Acknowledgements

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

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