A novel microRNA boosts hyper-β-oxidation of fatty acids in liver by impeding CEP350-mediated sequestration of PPARα and thus restricts chronic hepatitis C

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ABSTRACT

Imbalance in lipid metabolism induces steatosis in liver during Chronic hepatitis C (CHC). Contribution of microRNAs in regulating lipid homoeostasis and liver disease progression is well established using small RNA-transcriptome data. Owing to the complexity in the development of liver diseases, the existence and functional importance of yet undiscovered regulatory miRNAs in disease pathogenesis was explored in this study using the unmapped sequences of the transcriptome data of HCV-HCC liver tissues following miRDeep2.pl pipeline. MicroRNA-c12 derived from the first intron of LGR5 of chromosome 12 was identified as one of the miRNA like sequences retrieved in this analysis that showed human specific origin. Northern blot hybridization has proved its existence in the hepatic cell line. Enrichment of premiR-c12 in dicer-deficient cells and miR-c12 in Ago2-RISC complex clearly suggested that it followed canonical miRNA biogenesis pathway and accomplished its regulatory function. Expression of this miRNA was quite low in CHC tissues than normal liver implying HCV-proteins might be regulating its biogenesis. Promoter scanning and ChIP analysis further revealed that under expression of p53 and hyper-methylation of STAT3 binding site upon HCV infection restricted its expression in CHC tissues. Centrosomal protein 350 (CEP350), which sequestered PPARα, was identified as one of the targets of miR-c12 using Miranda and validated by luciferase assay/western blot analysis. Furthermore, reduced triglyceride accumulation and enhanced PPARα mediated transcription of β-oxidation genes upon restoration of miR-c12 in liver cells suggested its role in lipid catabolism. Thus this study is reporting miR-c12 for the first time and showed its protective role during chronic HCV infection.

INTRODUCTION

Chronic hepatitis C (CHC) is associated with complications like progressive development of liver cirrhosis (LC) and hepatocellular carcinoma (HCC) [1–3]. Persistent infection with hepatitis C virus (HCV) in liver is accompanied with immune mediated death of hepatocytes and aberrant wound healing, progressive liver fibrosis followed by neoplastic transformation. HCV infection follows an asymptomatic clinical course involving a variety of viral, host and environmental factors altering numerous host regulatory pathways [4]. With the advancement of recent genome-wide transcriptome profiling, several functional candidate target genes and pathways have been identified [5–8] which are associated to the HCV life cycle and development and progression of liver steatosis/fibrosis into cirrhosis and HCC following chronic HCV infection [9–11]. But the complexity of the crosstalk and interactions between different cellular and viral factors and their consequences leave numerous critical questions unanswered.

HCV mainly hijacks the genes of lipid metabolism pathways to favour its internalization, replication and assembly inside the hepatocytes [12,13]. Replication and infection of HCV is also tightly controlled by host regulatory RNAs such as small non-coding RNAs [14]. Among these regulatory RNAs, microRNAs (miRNA) are the most well studied 18–22 nucleotide long single stranded RNAs generated following canonical DICER-DROSHA mediated biogenesis pathway [15,16] and translationally repressed the target mRNA. MiRNome landscape analysis revealed that binding of hepatocyte specific miR-122 to the 5′UTR stabilizes HCV RNA while binding to the 3′UTR in HCV genome impair replication and translation of HCV [17,18]. In addition, genome wide small RNA transcriptome profiles have identified multiple miRNAs (miR-448, miR-199a, miR-181, miR-27, miR-130a/b, miR-122, miR-196), which are altered and play important role in HCV infection [17–21].

Interestingly, few such transcriptomic studies have emphasized on novel tissue specific and organism specific miRNA sequences retrieved from unmapped portion of the sequences and showed their importance in disease development. One such study in post mortem human brain led to identify 65
novel miRNAs and some of them having potential role in Huntington or Parkinson’s diseases [22]. Such novel miRNAs are also reported in chicken, human embryonic stem cells, solid cancers, etc. [23–26].

Here, we have explored the small RNA transcriptome profile of HCV-related HCC liver tissues and focused on the unmapped sequences to provide valuable insights about novel miRNAs and its impact on pathobiology of liver disease progression. Seven such novel sequences were retrieved after mapping of the sequences to 2700 known miRNAs of miRBase v.20 [27] and further characterized as miRNAs in this study. One of these miRNAs, miR-c12 originated from first intron of leucine rich repeat containing G protein coupled receptor 5 (LGR5) gene located on chromosome 12, was found specific to human liver only. Low-binding affinity of STAT3 due to hyper methylation of LGR5 promoter and downregulation of p53 expression upon HCV infection led to low expression of this miRNA in CHC patients. Over expression of this miRNA-induced peroxisome proliferator-activated receptor-α (PPAR-α) mediated hyper-fatty acid oxidation leading to low lipid and triglyceride accumulation in the hepatocyte and hence restricted HCV replication. Thus, miR-c12 plays important role in maintenance of the lipid homoeostasis in the liver and prevents hepatitis C virus infection.

Results
Over the last three decades, myriads of miRNAs were identified and characterized to unveil the pathophysiology of various cancers. To search such deregulated miRNAs in HCC next generation sequencing (NGS) followed by bioinformatics analysis was employed using the liver tissue sample of HCC patients and normal individual. The unified ID of the submitted NGS data in the GEO database is GSE140370. During alignment of the data to the annotated miRNA sequences in the miRBase v.20, approximately 20% of the reads remain unmapped. Considering the complex mechanism of liver disease progression, these unmapped sequences were explored further to identify candidate sequences having potential to behave as miRNA. Thus, the unmapped reads were subjected to miRDeep2.pl algorithm and seven sequences which follow the prediction criteria for miRNAs and present in each of the sample subjected to NGS analysis were retrieved using MFOLD web server. These sequences were also aligned with the miRNA database of the other species and six sequences were found conserved among various species (data not shown) while one sequence was noted exclusively as human specific miRNA sequence. To further validate and to determine the precursor coordinate of all probable mature miRNAs, the reads were subjected to Randfold tool and were referred according to the chromosome number (Supplementary Table 1).

The human specific miRNA, miR-c12, which belong to chromosome 12 having A-U content 61% was characterized further here (Fig. 1A). This miRNA was found to be originated from the first intron of the leucine-rich repeat containing G protein–coupled receptor 5 (LGR5), a stem cell marker [28] (Fig. 1B).

Experimental validation of novel miRNAs in multiple cell lines and in DICER-depleted hepatoma cells
The intracellular miR-c12 was evaluated first by northern blot analysis using Huh7 cellular RNA. The premiR-c12 sequence was cloned and overexpressed in Huh7 cell line as positive control while total RNA was used as loading control (Fig. 1C). The expression of mature miR-c12 was further verified in Dicer knock down cells with shRNA pSiCoR-DICER1 (Addgene#14,763) [29] using stem-loop-RT-qPCR (SLRT-qPCR), Low expression of mature miR-c12 and enrichment of premiR-c12 suggested that synthesis and maturation of this miRNA followed canonical miRNA biogenesis pathways (Fig. 1D) and it was not an intron degradative product. MicroRNA-21 and miR-122 was considered as positive control for DICER processing. Western blot analysis with anti-Dicer antibody showed the knock down of the protein after 48 h of transfection (Fig. 1E).

Next to determine its’ association with RNA induced silencing complex (RISC), Huh7 cells were co-transfected with pAgo2-Flag and pPre-miR-c12. The RNA-immunoprecipitation (RIP) was performed with anti-Flag antibody after 48hrs of transfection while anti-mouse-IgG antibody was used as negative control. The SLRT-qPCR data showed that miR-c12-3p was loaded on RISC to target complementary mRNA following canonical pathway (Fig. 1F).

Next to verify its organ specificity expression of miR-c12 was evaluated in liver and non-liver cell lines by SLRT-qPCR. A comparable expression pattern was observed in Huh7/ Huh7.5 (liver cancer), HCT116 (Colon cancer) and MDA-MB-231 (Breast cancer) cell lines while HEK-293 (Normal Kidney) cells showed minimal expression (Fig. 1G). Interestingly, expression of this miRNA was very poor in LX2 (primary liver fibroblast cell) and HUVEC (Human umbilical vein endothelial cell) ratifying its hepatocytic origin. Multi-tissue cDNA panel analysis also showed that expression of miR-c12 was highest in the liver compared to other organs (colon, oral and stomach) (Fig. 1H).

Expression pattern of miR-c12 in HCV- and HBV-infected liver tissues with progressive disease stages
The expression pattern of miR-c12 was further verified in HCV infected liver tissue samples with progressive liver disease stages such as chronic hepatitis C (CHC), liver cirrhosis (LC) and HCC. Normal liver tissue was used as control. The expression of miR-c12 was found significantly downregulated in CHC tissues (n = 8) compared to normal while it was gradually increased in HCC through LC (Fig. 2A). A similar pattern of expression was observed for LGR5 and premiR-c12 in CHC liver tissues and in either HCV-cc or HCV-core transfected Huh7 cell line (Fig. 2B–D) implicating HCV-core protein might be commonly regulating the expression of the two transcripts. No such significant regulation in expression of miR-c12 was noticed in HBV infected liver tissue specimens and HBV transfected cell lines (Supplementary Figure 1a and 1b). Expression of HCV negative strand RNA and HBx gene was quantified to verify positive HCV and HBV infection in vitro.
Figure 1. Prediction and validation of miR-c12 as novel miRNA. (A) Stem-loop structure of the precursor sequence of miR-c12. (B) Chromosomal position of the novel miRNA-c12 was in the 1st Intron of LGR5 gene. P53 and STAT3 were two transcription factors regulated by HCV proteins and bound to LGR5/miR-c12 promoter at −985nt and −345nt position where CpG island was also present. Expression of miR-c12 verified by (C) northern blot analysis with anti-miR-c12 probe using Huh7 cellular RNA. Over expressed PremiR-c12 and intracellular miR-122 was used as positive control, (D) expression of miR-c12 and premiR-c12 in Dicer Knockdown cells where miR-21 and miR-122 was used as positive miRNA synthesized following canonical biogenesis pathway, (E) Western blot analysis with anti-Dicer antibody to show downregulation of Dicer upon shRNA treatment. (F) RNA immunoprecipitation assay with anti-Ago2 and anti-iG antibody after transfection of premiR-c12 construct and control vector in Huh7 cells. Expression analysis of miR-c12 in (G) different cell lines and (H) different normal tissues. *p < 0.05, **p < 0.01 and ***p < 0.001 were considered significant and represented as *, ** and ***, respectively.

Transcriptional co-regulation of the promoter of LGR5 and miR-c12

To understand the transcriptional regulation of the LGR5 promoter upon HCV infection, the promoter region of LGR5 was scanned using TFSEARCH, TFBIND and also explored the ChIP-Seq data in UCSC Genome Browser along with the previous literatures. Interestingly, binding sites of P53 and STAT3 was noted at −985nt and −345nt position in the promoter region of LGR5 gene, respectively (Fig. 1B). Hence, p53 and STAT3 expression was verified in HCV-infected cell line (Fig. 2C). To our surprise, although expression of p53 was noted down in HCV-infected cell line, STAT3 expression level was higher compared to control cells (Fig. 2C). ChIP assay with anti-p53 antibody also showed less binding of p53 to the LGR5 promoter upon HCV infection (Fig. 2E) while the expression of miR-c12 was restored upon overexpression of p53 (Fig. 2F). On further analysis of the LGR5 promoter a stretch of CpG island was noted in the binding site of STAT3 region which was also reported earlier in the literature [30]. Thus expression of the three transcripts (miR-c12, premiR-c12 and LGR5) were verified after treatment with 5-azacytidine (Fig. 2G). Furthermore, ChIP analysis with anti-STAT3 antibody after 5-azacytidine treatment and HCV infection showed more binding of STAT3 to the LGR5/miR-c12 promoter (Fig. 2H). These data suggest that HCV infection restricts binding of p53 and STAT3 to the LGR5/miR-c12 promoter by reducing P53 expression and inducing CpG methylation at STAT3 binding site, respectively.

Prediction of target genes to investigate the protective role of miR-c12

To understand the biological relevance of miR-c12 in liver tissue, target genes were predicted using miRanda algorithm. Fifty-four target genes were identified considering complementarity >6 nucleotides, minimum free energy (MFE) ≤ 20 Kcal/mol and score ≥140. Molecular interactive network of miRNA and mRNA or targetome were determined using Ingenuity pathway analysis (IPA) which revealed that target genes were involved in intercellular signalling, liver steatosis and fibrosis, immune cell trafficking and carcinogenesis pathways (Fig. 3A). Out of nine target genes verified after in vitro transfection of miR-c12 in Huh7 cells, expression of six genes (CEP350, SORT1, FBN1, HNRNPC, PDK1 and MARK2) were found significantly down-regulated when miR-c12 was overexpressed compared to control.
These genes are mostly related to HCV infection, lipid metabolism and carcinogenesis pathways (Fig. 3B). We further validated five target genes (SORT1, ABCA1, RAF1, PDPK1) from lipid metabolism pathways in normal and CHC liver tissues. All the four target genes were upregulated in liver tissue of CHC patients where miR-c12 was downregulated compared to normal (Fig. 3C).

The role of miR-c12 in fatty acid oxidation and signalling pathways

Now to understand the role of miR-c12 in HCV infected liver diseases, first its’ impact on HCV infection was verified upon over expression of miR-c12 by quantifying HCV RNA after 48 h of HCV infection as described in Materials and methods. Interestingly, the negative strand of HCV RNA was reduced significantly in this assay while anti-miR-c12 treatment rescued it partially (Fig. 3D) implicating this miRNA has potential to restrict HCV infection in liver cell line.

To investigate the pathways underlying the protective role played by miR-c12, targets of miR-c12 were revisited and found that one of the targets of this miRNA was Centrosomal protein 350 (CEP350) gene, which negatively interacts with ligand activated transcription factor, peroxisome proliferator activated receptor alpha (PPARα) and reduce its nuclear trans-localization or restricts its transactivation property [31]. Binding of miR-c12 to the 3’UTR of...
CEP350 was verified by luciferase-reporter assay with increasing doses of miR-c12 (Fig. 3E) using wild type and mutant 3′-UTR constructs. A negative correlation between the expression of CEP350 and miR-c12 in liver tissues has further confirmed their association (Fig. 3F).

Next, expressions of few β-oxidation genes of lipid metabolism pathway such as CPT2 and ACOX1, which have PPARα binding sites in the promoter region, were determined in Huh7.5 cell line infected with HCV and a significant over production of both the genes was noted upon restoration of
mir-c12 and anti-CEP350 oligo treatment (Fig. 4A). Higher binding ability of PPARα to the promoter of these genes were confirmed by ChIP assay with anti-PPARα in presence of excess mir-c12 (Fig. 4B). Similar downregulation of ACOX1 and CPT2 was observed in CHC tissue where expression of mir-c12 was lower and CEP350 was higher in comparison to normal liver (Fig. 4C).

Furthermore, western blot analysis with nuclear fraction of miR-c12 overexpressed and anti-CEP350 oligo treated Huh7 cells showed restoration of more PPARα in the nucleus compared to vector while anti-mir-c12 treatment showed opposite data. No significant difference in protein level of PPARα was noticed in total cell extract (Fig. 4D and E). The signalling of PPARα occurred through ERK1/2 pathways (Fig. 4D) while AKT was downregulated as it is a direct target of miR-c12.

**Clinical implication of overexpression of mir-c12 in liver**

In clinical specimens, expression of this miRNA was noted lower in liver tissue of CHC patients than in normal as shown earlier (Fig. 2A) implicating this miRNA plays protective role in normal liver. The enhanced expression of this miRNA in HCC compared to CHC is under investigation (Fig. 2A). Similar to other reports [32], triglyceride (TG) level was found lower in the serum of CHC patients compared to normal as it is accumulated in the liver tissue due to low oxidation of fatty acids (Fig. 5A). On the other hand, intracellular TG level was found low in mir-c12 over expressed and in anti-CEP350 oligo treated Huh7 cells while TG was raised upon anti-mir-c12 treatment as shown by colorimetric analysis and Oil-O-red staining (Fig. 5B and 5C). Hence, a positive correlation was noted between the expression of mir-c12 and serum TG level (Fig. 5D).

Thus, this study suggests that the novel microRNA miR-c12 induces lipid metabolism by impeding cytoplasmic CEP350 and inducing transcriptional activity of PPARα in the nucleus. This triggers fatty acid β oxidation and reduces accumulation of fat in the liver that in turn restricts HCV infection as HCV lifecycle is intimately connected to lipid metabolism (Fig. 5E).

**Discussion**

To understand myriads of questions in biology including complexity in cancer, next generation sequencing (NGS) has made great strides by digitally analysing the genetic material at complete genome scale. It provides a better understanding of molecular pathogenesis of a disease by identifying known as well as rare undefined novel and low abundant sequences present in the same sample. This high throughput technology has been utilized to understand the biology of HCV pathogenesis. But lack of functional genomic attempts to use the vast amount of high density data generated in genomic and transcriptomic analysis, knowledge of various pool of RNAs including non-coding regulatory RNAs and proteins are not adequate to understand the disease biology well [33,34]. Thus a novel integrated approach was considered in the current
study emphasizing on the unmapped sequences obtained after repeated alignment of the sequences to the human genome and retrieved low copy novel candidate miRNAs having functional corroboration with disease development.

In our study, small RNA transcriptomics data of HCV related HCC and normal individuals were analysed and mapped to the miRbase V.20 followed by unmapped reads were mined to discover novel liver specific miRNAs. Thus, following the widely accepted set of criteria for delineation of mature miRNA [35], seven sequences were endorsed as miRNAs and considered for further validation. Given the importance of NGS technologies on RNA research, a parallel platform such as Ion Torrent PGM was also used to abolish any artefacts and to reconfirm such novel data (data not shown).

One of these seven novel microRNAs, miR-c12 was discovered as human specific miRNA which was originated from the

Figure 5. Effect of miR-c12 and CEP350 on serum and intracellular triglyceride level: (A) Serum Triglyceride content in normal individuals vs. CHC patients. (B) Intracellular triglyceride content in HCV infected Huh 7.5 cells transfected with miR-c12, anti-miR-c12 and anti-CEP350 oligo. (C) Oil-Red-O staining of HCV infected Huh 7.5 cells in presence or absence of miR-c12. (D) Correlation of miR-c12 expression and serum triglyceride level in liver tissue. (E) Schematic diagram of the miR-c12-CEP350-PPARα axis in regulating transcription of β-oxidation genes. p values <0.05, <0.01 and <0.001 are represented as * and **, respectively.
first intron of LGR5 gene located in chromosome 12. Intracellular presence of this miRNA was confirmed by northern blot analysis followed by expression analysis in liver cancer cell lines and in normal liver. Canonical biogenesis and functional competency of the miRNA was verified by DICER knockdown assay and Ago2-RNA immunoprecipitation assay.

To investigate the contribution of this miRNA in liver disease biology including HCV infection, expression pattern in chronic HCV-infected liver tissues, its transcriptional regulation and functional correlation with liver disease development were studied. It is reported by several groups that the expression of various miRNAs are downregulated by HCV proteins in order to facilitate its own replication [14,36]. The expression pattern of miR-c12 was also significantly reduced in chronic HCV infected liver tissues, and in Huh7 cells infected with HCV. This expression pattern was very similar to the expression of LGR5 while inverse to the expression of HCV core protein implying HCV-core mediated co-transcriptional regulation in their expressions. The promoter region of LGR5 has binding sites for two important HCV-regulated transcription factors such as p53 [37] and STAT3 [38]. P53 is a tumour suppressor gene it is repressed upon HCV infection as cellular protein kinase R (PKR) is activated by HCV RNA and suppresses global protein synthesis in infected cells [39]. In addition, HCV NS5A interacts with p53 thereby reducing its accessibility [40,41]. We have also found that overexpression of p53 has enhanced the transcription of LGR5/premI-R-c12 while HCV infection has reduced the expression of p53.

A controversial data is available about STAT3 expression in HCV infected cells. Stevensonet al. have shown that HCV core protein downregulates STAT3 protein by activating its proteosomal degradation [42] whereas, upregulation of STAT3 in HCV infected cells is also reported by several groups [43,44]. An upregulation of STAT3 expression was observed in Huh7 cells infected with HCV in our study. Su et al. in 2015 have shown that the level of LGR5 in cancer cell is regulated by methylation pattern of the CpG island in the promoter [30]. One CpG island was noted in the STAT3 binding site of LGR5/premI-R-c12 promoter and thus anti-STAT3 ChiP analysis after treatment of demethylation reagent 5-Azacytidine led to overproduction of LGR5 and miR-c12 in both presence and absence of HCV infection. Although the expression analysis of LGR5 gene in the available methylome data (GSE82177) with small number of HCV infected non-tumour (n = 8) and un-infected control (n = 9) revealed no significant (p value = 0.08) downregulation, HCV infected non-cancerous tissue with either LC/CH background (n = 5) vs. HCC tumour (n = 5) methylome data (GSE19665) showed lower methylation and significant overexpression of LGR5 in HCC. Lei et al. had also showed recently that Histone Methylase, Lysine specific demethylase 1 (LSD1) induced high expression of LGR5, is associated with chemoresistant and poor prognosis of HCC [45].

To understand the biological relevance of low expression of miR-c12 in CHC and its progression towards HCC, target genes were predicted and explored the molecular interactive network of miR-c12 using IPA. It revealed that target genes are involved in Hepatitis C infection (PDK1, RAF1, AKT1, IFNAR1, HNRNPC), lipid metabolism (ABCA1, SORT1, ACADL, PDK1, CEP350), insulin resistance (PDK1, RAF1, AKT1, FOXO1, IRS2), apoptosis (PDK1, RAF1, AIFM1, DEFA, CFLAR) and in cancer-related pathways (FOXO1, IFNAR1, NOTCH2, RAF1, TGFβR2, WNT16).

Centrosome associated protein 350 or CEP350, one of the lesser studied proteins showed restricted expression with increasing doses of miR-c12 in cell line and 3'UTR assay showed direct interaction between these two partners. Patel et al. have reported that in presence of CEP350, diffusely present nuclear PPARa co-localizes with CEP350 in the perinuclear region and in the centrosome or with the intermediate filaments. This restricts the function of PPARa which regulates gene expression in conjunction with activated retinoid X receptor (RXR) binding to the cis-peroxisome proliferator responsive element (PPRE) of the target gene [31]. Hence, impeding the expression of CEP350, the miR-c12 has triggered the PPARa mediated activation of PPRE containing fatty acid β-oxidation enzymes such as CPT2, ACOX1, and enhanced fat catabolism in the hepatocyte, which in turn restricted replication of HCV in hepatocyte [46]. Bojana Rakic, et al. in 2005 has showed antagonist of PPARa inhibits HCV replication [47] while Shirasaki, et al. has showed the regulation of lipid metabolism and HCV replication by miR-27a which target RXR and ATP binding cassette transporter ABCA1 [48].

Role of PDK1, AKT1, RAF1 and HNRNPC in establishment of HCV infection has been described in several studies [49]. miR-c12 also targets PDK1 and RAF1, both are known to interact and stabilize HCV replication complex [50,51] whereas HNRNPC interacts with pyrimidine-rich region of HCV 3' UTR and initiates HCV RNA replication [52]. Among various signalling molecules, AKT of P13K-AKT signalling pathway which enhances HCV encoded protein translation in absence of viral 3'UTR by activation of SREBP1 and SREBP2 was also targeted by miR-c12 [53].

On the other hand, low expression of ABCA1, a lipid transporter targeted by miR-c12, reduced efflux of triglycerides (TG) and cholesterol (CHO) to lipid poor apolipoproteins and helping in HDL synthesis. While low level of trans Golgi network protein Sortilin 1 (SORT1) in presence of excess miR-c12 reduces VLDL biosynthesis and release, which in turn reduces extracellular and intracellular infectious viral titres by inhibiting assembly of HCV [32].

Thus, this study highlights that 20–25% unmapped sequences found in the NGS analysis probably due to gaps in the reference genome. This unmapped data could also provide important information to comprehend the disease biology. Here, realignment of the unmapped sequences to the genome led to discover of a novel human specific miRNA that has high potential to target multiple genes from lipid metabolism and trafficking pathways that account for the progression of liver diseases towards HCC upon HCV infection, hence this miR-c12 could be used to improve the outcome of the anti-HCV therapy, particularly, in direct acting antiviral therapy resistant patients and patients with severe steatosis.
Materials and methods

Ethics statement

Ethics committee of the Institute of Post Graduate Medical Education and Research (IPGME&R), Kolkata, India and Indraprastha Apollo Hospital, New Delhi had approved the study (Approval ID: Inst/IEC/2015/447; dated 07 July 2015). Blood and tissues were collected from patients who were willing to participate in the study with prior written consent.

Study subjects

Mono-infected chronic hepatitis C and hepatitis B patients visiting Hepatology outdoor of two centres, Institute of Post Graduate Medical Education and Research (IPGME&R), Kolkata, India and Apollo Hospital, New Delhi, India for evaluation of their liver diseases from 2015 to 2018 and having age between 18 and 75 years were included in the study. Patients co-infected with other hepatitis viruses, positive for anti-HIV, chronic alcoholics and unwilling to comply with the study were excluded.

Out of 59 patients \( (n_{\text{HCV}} = 33, n_{\text{HBV}} = 26) \) 17 patients \( (n_{\text{HCV}} = 9, n_{\text{HBV}} = 8) \) clinically diagnosed with liver cirrhosis (LC) as they had oesophageal or gastric varices, portal hypertension, splenomegaly/dilated portal vein with altered flow pattern, ascites, etc., 27 patients \( (n_{\text{HCV}} = 16, n_{\text{HBV}} = 11) \) were found to have HCC by standard protocols of triphasic CT scan and/or AFP value \( (>250 \text{ ng/mL}) \) (cut-off value for Indian HCC patient) [54]. Histological evidences had further confirmed each case. Fibroscan was used to measure tissue elasticity and value \( <7 \text{ kpa} \) was taken as no fibrosis while \( >14 \text{ kpa} \) as LC [55]. Fifteen chronic hepatitis \( (n_{\text{HCV}} = 8, n_{\text{HBV}} = 7) \) was confirmed from clinical, radiological and histological evidences. The indication for biopsy of chronic hepatitis B (CHB) patients were with high ALT, HBV DNA level \( >10^5 \), HBV e-Antigen positivity with inconclusive fibroscan report while chronic hepatitis C (CHC) patients with high ALT and with inconclusive fibroscan report or older age were subjected to biopsy.

Liver tissues were also collected from gall bladder carcinoma (GBC) patients \( (n = 15) \) attending gastrointestinal surgery clinic of IPGME&R, Kolkata and subsequently undergone undergone cholecystectomy as routine analysis for verification of liver metastasis. After histological verification, nine subjects who were negative for HBV, HCV and HIV having normal liver architecture were included as control.

Total RNA isolated from adjacent non-tumour region of Oral and Stomach cancer were kindly provided by Prof Bidyut Ray, ISI, Kolkata; and Dr Shalini Datta, ISI, Kolkata, respectively. Normal colonic tissues were collected from the hemorroid patients who were subjected to colonoscopy in the Department of Gastroenterology, IPGME&R, Kolkata and routine biopsy was taken for diagnosis.

All the liver tissues were collected in RNA later (Ambion, USA) and in 10% formalin immediate after surgery at room temperature. Tissues collected in RNA later was kept at 4°C for overnight and then preserved at −80°C for future use. About 6 mL blood was collected either in presence or absence of anti-coagulant for biochemical assays.

Biochemical parameters

Each sample was subjected to blood biochemistry such as ALT, AST, total protein, albumin, globulin, bilirubin, prothrombin time (INR) using commercially available kits from Bayer Diagnostics, India. Serological markers such as HBsAg, anti-HCV anti-HIV were verified using ELISA kits from General Biologicals, Taiwan and Biomerieux Boxtel, The Netherlands. Demographical, clinical and histological data of each patient are presented in Supplementary Table 2.

Isolation of total RNA

After crushing in liquid nitrogen, 3 mg of each liver tissue was taken to isolate total RNA using TRIzol (Invitrogen, USA) following manufacturer’s instructions. RNA integrity was determined in Agilent Bioanalyzer and RNA integrity (RIN) values between 7 and 10 were considered for further analysis.

Next generation sequence analysis and identification of novel miRNA

Small RNA sequencing was performed with liver tissues of normal \( (n = 3) \) and HCV-HCC \( (n = 3) \) of read length \(<40\) nucleotides on Illumina Platform HiSeq 2500. The methodological detail of NGS was followed as described previously [56] and the sequencing data is provided in GEO dataset (ID: GSE140370).

After removing the low quality reads and the adapter sequences masking the clean reads, the filtered sequences were aligned to the hg19/GRCh37 human genome reference sequence using mapper.pl of the miRDeep2 module followed by alignment with the miRNAs of related species by miRDeep2.pl. The putative sequences were aligned with rRNA, tRNA, snRNA, snoRNA and piRNA sequences using NCBI BLAST software and mapped sequences were removed. Subsequently on the basis of the following criteria novel miRNA were predicted: 1) the stem-loop structure formation by the precursor sequences with minimum free energy (MFE) cut-off less than −18 kcal/mol predicted by Randfold and mFold, 2) A-U content of the sequence cut-off being 30–70%, 3) present in all biological replicates, and 4) read count being >10. Targets of the novel miRNA were predicted using MiRanda by scanning the sequences of the 3’ UTR regions of human genes considering complementarity of at least 6 nucleotide having MFE less than −20 kCal/mol and score more than 140.

Real time quantification of miRNA and mRNA by qRT-PCR

To quantify miRNA and mRNA in liver tissues by qRT-PCR, cDNA was prepared separately using miScript PCR Starter Kit (Qiagen) and RevertAid RT Reverse Transcription Kit (Thermo Scientific), respectively, following manufacturer’s instructions. Quantitative PCR was performed using SYBR Green Real-time PCR Master Mix (Roche Diagnostics) in ABI Quant Studio 7 Flex Real Time PCR System. Sequences of each primer are presented in Supplementary Table 3.
Relative expression levels were calculated using ΔCt method, where ΔCt = Ct (miRNA or mRNA of interest) – Ct (internal control gene). RNU6B and 18 S rRNA were used as internal control for quantification of miRNA and mRNA, respectively. Each experiment was performed in duplicates and twice.

**Northern blot hybridization**

About 20 mg of total RNA was separated on a 15% UREA–polyacrylamide gel, transferred to Immobilon-PVDF membrane (Millipore) and cross-linked under UV radiation. Hybridization was carried out following standard protocol. Anti-miR-122 sequence was labelled with γ-P32 and used as probe while LNA probe was used in case of anti-miR-122. The experiment was repeated twice.

**Cloning of premiRNA and 3′UTR of the target gene**

PremiRNA and 3′UTR of the target gene was cloned in pRNAU6.1/neo and pSiCHECK2 (Promega) vectors, respectively. Prof Nitai P Bhattacharyya, Saha Institute of Nuclear Physics, Kolkata had kindly gifted the pRNAU6.1/neo-vector.

**Transfection and luciferase assay**

The quality and integrity of human hepatoma cell lines Huh7 and Huh7.5 was verified by short tandem repeat (STR) DNA profiling (LifeCode Technologies Private Limited, India). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS) ( Gibco, USA) and 100 units/mL penicillin-streptomycin (Sigma Aldrich, Germany) in a 5% CO2 containing 37°C humidified chamber. Plasmid DNA was transfected using Lipofectamine 2000 (Thermo Scientific) following the standard manufacturer’s protocol and the expression of the target genes were determined after 48 h by qRT-PCR. Luciferase assay was performed using Dual Luciferase Reporter assay kit (Promega) following manufacturer’s protocol. Each experiment was repeated twice.

**In vitro HCV RNA transcription**

The cDNA clone of HCV genotype 3 (S52/JFH1) was linearized by restriction digestion with Xba1 enzyme (NEB) and the 3′ single strand overhang was removed with Mung Bean Nuclease (NEB). The linearized plasmid DNA was precipitated with ethanol and then in vitro transcribed into RNA using MEGAscript T7 Transcription kit (Thermo scientific). RNA was purified using Trizol and integrity was verified on RNA gel.

**HCV infection assay**

HCV RNA was transfected in 4 × 10⁴ Huh 7.5 cells seeded in a 24 well plate using lipofectamine 2000 (Thermo Scientific). Mock transfected well was used as negative control. After 48 hrs, cell culture conditioned media containing virus particles (HCVcc) was filtered through 0.45-μm-pore-size cellulose acetate membrane and preserved at 4°C for future use. To determine the dilution of infection, 1 × 10³ Huh7.5 cells seeded on 96 well plate was infected with serially diluted media and cell proliferation was determined using WST1 reagent (Sigma-Aldrich). Dilution at which more than 50% of cells survived was selected for infection assay. Thus, 1 × 10³ Huh7.5 cells seeded in 96 well plates were first transfected with pPre-miR-c12 and control vector and then the media containing HCVcc was added to infect cells at a dilution of 1:1000. Trypan Blue exclusion assay and WST1 assay was used to count live cells. After 72 h total RNA was isolated and expression of HCV RNA, miRNA and different genes were determined. HCV RNA was quantified by X-Tail PCR using the previously described procedure [57].

**Immunoblot analysis**

Isolation of total protein extract was performed using RIPA buffer following the standard protocol. In case of nuclear protein isolation, first the cytoplasmic protein was separated using mild lysis buffer (150 mM NaCl, 50 mM TrisHCl, 0.5% Sodium Deoxycholate, 0.1% SDS, 1% NP-40) followed by lysis with increased salt concentration (50 mM HEPE (pH 7.4), 150 mM NaCl, 10 mM KCl, 5 mM MgCl2, 2% NP-40). Protease inhibitor cocktail (Thermo Scientific) was added in each lysis buffer. Total cell lysate was separated on a SDS-PAGE gel, transferred on PVDF membrane and incubated with the following antibodies as required: anti-PPARα (Novus Biologicals, CO, USA), anti-ERK1, anti-phospho-ERK1, anti-phospho-ERK1/2 (Cell Signalling, Massachusetts, USA); anti-α-Tubulin (Sigma, Germany). The HRP-conjugated secondary antibodies (cell signalling) were used to identify the protein of interest. Each experiment was repeated at least two times.

**RNA-Immunoprecipitation (RIP)**

Ago2-Flag plasmid (Addgene#21,538) [58] was co transfected with control vector and premiR-c12 plasmid in Huh7 cells. Two days after transfection, cells were harvested in ice cold freshly prepared RIP buffer (150 mM KCl, 25 mM Tris [pH7.4], 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, RNase inhibitor [Thermo Scientific], Protease inhibitor [Thermo Scientific]). Protein was estimated using Bradford reagent (Sigma) and equal amount of protein was used for immunoprecipitation with anti-Flag (2 μg) and non-specific anti-IgG2 antibody and incubated overnight at 4°C with gentle rotation. Tubes were incubated another 2 h with Protein A/G beads (Sigma) and then pelleted beads at 2500rpm for 30 sec and washed 3 times with RIP buffer. RNA was isolated from the beads using Trizol following manufacturer’s protocol and quantifies the miRNAs as discussed earlier. The data was presented after repeating the experiment two times.

**Chromatin immunoprecipitation (ChIP)**

Huh7 cells were transfected with control vector, pPre-miR-c12 and HCV-RNA for 48 hrs independently and then cross linked using formaldehyde and quenched with glycerine followed by sonication to shear DNA to an average length of 200–1000 bases. Supernatant was purified, and incubated with
antibodies (anti-PPAR-α, anti-P53, anti-STAT3 and anti-IgG separately) for overnight in a rotor. Chromatin was precipitated with Protein A/G beads, washed with ChIP buffer and DNA was eluted by Phenol-Chloroform method. Cells were first treated with 5 μm of 5-Azacytidine (Sigma) before transfection for demethylation study. Experiment was repeated twice.

**Oil red-O staining and estimation of triglyceride (TG) content**

Huh7 cells transfected with pPremiR-c12, anti-CEP350 oligo and control vector separately were fixed with 10% formalin for 30 min. Then the cells were washed with PBS and incubated in 60% isopropanol for 5 min. Isopropanol was removed and stained with Oil Red O Solution (0.5 gm dissolved in 100% isopropanol and filtered) for 20 min. The solution was washed off with distilled water and viewed under light microscope (EVOS XL Core cell imaging system, Life technologies). The intracellular stain was then extracted in isopropanol overnight and quantified in a 96 well plate reader at 492 nm.

TG content in serum samples and intracellular Huh7 cells transfected as described earlier were quantified using Autozyme Triglyceride colorimetric assay kit following manufacturer’s protocol (Accurex Biomedical Pvt. Ltd.). The Huh7 cells were lysed with RIPA buffer followed by treatment with the reagent and the colorimetric reading at 510 nm was normalized with the cellular protein content.

**Statistical analysis**

The differential expression analysis of miRNAs in liver tissues of HCC patients compared to control were performed using DESeq package in R software. GraphPad Prism 5 (GraphPad Software, USA) was used for statistical analysis. Student’s t-test was used to compare the in vitro data and ANOVA test was performed to compare among the normal, CHC, LC and HCC groups. The qRT-PCR data is presented as mean and mean standard error. p < 0.05 was considered as statistically significant.

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**Authors contribution**

S. Banerjee and S. Ghosh had conceived idea, analysed data, prepared the manuscript. S. Banerjee has full access to all the data and had final responsibility for the decision to submit manuscript for publication. S. Bhowmik, A. Ghosh, P. Kumari collected samples, and isolated RNA for real-time validation. S. Roy, A. Goswami, S. Bhattacharyya did Oil-Red-O stain and Northern blot analysis. S. Dokania did the site directed mutagenesis of 3’-UTR clone of CEP350. S. Ghosh, J. Chakraborty and R. Chatterjee did the analysis of NGS data and S. Datta, A. Chowdhury read the paper carefully.

**Disclosure statement**

The authors declare no conflict of interest.

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**Data availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request. The sequencing data used for novel miRNA identification is provided in GEO dataset GSE140370.

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