Sponging of five tumour suppressor miRNAs by IncRNA-KCNQ1OT1 activates BMPR1A/BMPR1B-ACVR2A/ACVR2B signalling and promotes chemoresistance to hepatocellular carcinoma

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Keywords: MicroRNAs, Hepatocellular Carcinoma, Convergent miRNAs, IncRNA, KCNQ1OT1

Posted Date: July 12th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3043406/v1

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Abstract

Diverse mechanisms have been established to understand chemoresistance of hepatocellular carcinoma (HCC), but the contribution of non-coding RNAs are not surveyed well. We aimed here to explore the lncRNA/miRNA axis in hepatitis C and B virus infected HCC to investigate the mechanism of chemoresistance and to classify a potential therapeutic target for HCC.

The small RNA transcriptome, and qRT-PCR validation with the liver tissues of both HCV and HBV infected HCC patients revealed that miR-424-5p/miR-136-3p/miR-139-5p/miR-223-3p/miR-375-3p were the most downregulated five miRNAs in HCC compared to normal (log\textsubscript{2} fold change ≤ -1.5, \textit{P\textsubscript{adj}} ≤ 0.05). \textit{In-silico} pathway analysis with the validated targets of each miRNA depicted that the signalling pathways regulating pluripotency of stem cells commonly targeted by the all five miRNAs. Subsequent validation by 3'UTR-luciferase assays and western blot analysis disclosed that these five miRNAs impeded either same or diverse genes, but all from BMP signaling pathways including BMPR1A/BMPR1B by miR-139-5p, miR-136-5p & miR-375-3p and ACVR2A/ACVR2B by miR424-5p & miR223-3p. Furthermore, restoration of each miRNA in Huh7/SNU449 cells inhibited phosphorylation of downstream SMAD1/5 and ERK1/2, and attenuated EMT/stemness/sphere formation/chemoresistance/invasion/migration of cells.

To investigate the mechanism of suppression of these miRNAs, “DIANA” prediction tool was employed and lncRNA-KCNQ1OT1 was retrieved as interacting partner of all five miRNAs. Ago2-RNA-immunoprecipitation/in vitro RNA pull-down assays revealed that lncRNA-KCNQ1OT1 physically interacted and sequestered all the five miRNAs in the cytoplasm. Hence, KCNQ1OT1 was deleted in Huh7/SNU449 cells using CRISPR-technology and observed regression of oncogenic properties with enhanced chemosensitivity and reduced metastasis of cells. Shrinkage of tumor size/volume in NOD-SCID mice injected with KCNQ1OT1-K/O cells further strengthened our observations.

Thus, lncRNA-KCNQ1OT1 is the main regulator, which reduces the level of beneficiary miRNAs in the tumor milieu and modulates BMP signaling to promote chemoresistance to HCC suggesting lncRNA-KCNQ1OT1 might have robust potential to be a therapeutic target for HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most aggressive primary liver cancers accounting for the 3rd highest cancer related mortalities worldwide [1, 2]. Decades of chronic infection with hepatitis B and hepatitis C virus (HBV or HCV) are the major risk factors for HCC [3]. Unfortunately, curative therapy can only be given to a minority of HCC patients. The complex molecular heterogeneity is the major hurdle in offering therapy to advanced HCC patients who are often chemo-resistant [4, 5]. Sorafenib is the only approved drug that could extend life expectancy of these patients for 4–6 months [6]. This evokes the necessity of identification of new promising therapeutics and also research the pathophysiological mechanisms to improve therapeutic strategy. The cancer stem cell (CSC) like features of cancer cells are responsible for such chemo-resistance [7]. Hence, molecular level understanding of pathways driving CSC
like features are required to identify effective therapeutics for HCC. In various cancers, several studies have implicated the importance of bone morphogenetic proteins (BMPs) during carcinogenesis by modulating proliferation, invasion and metastasis [8]. In this context, the secreted cytokine BMP4 and BMP2 have been shown to function through type 1 receptor BMPR1A/BMPR1B and type 2 receptor ACVR2A/ACVR2B [9]. Chiu CY et al in 2012 has established that BMP4 and BMPR1A type I receptor promotes proliferation and metastasis in HCC through SMAD1/5/8 independent ERK1/2 pathway [10]. BMP4 and its receptor BMPR1A are overexpressed in various cancers including HCC and promotes CSC like features such as expression of CD133 marker and metastasis [11–13].

Non-coding RNAs (ncRNAs) including both microRNAs (miRNAs, 18-22nt long) and long non-coding RNAs (lncRNAs, >200nt long) have garnered attention as major epigenetic regulators of almost all cellular pathways [14]. Several groups have constructed disease specific differentially expressed miRNA-lncRNA-mRNA regulatory network [15] but less data available on BMP signalling. Li Lian et al in 2015 have characterized aggressive HCC with active hsa-miR-148a-ACVR1-BMP-Wnt circuit [16].

In this study, we provide for the first time significant molecular information to support the regulatory network of mRNAs-miRNAs-lncRNA in BMP signalling for maintenance of CSC like features of HCC cells. Five most downregulated tumour suppressor miRNAs (miR-424-5p/miR-136-3p/miR-139-5p/miR-223-3p/miR-375-3p) in HCC liver were retrieved from our small RNA transcriptome data. Surprisingly, target recognition and pathway analyses revealed that these five miRNAs commonly impeded pluripotency of stem cell signalling pathways where their targets were BMP receptors, BMPR1A/1B and ACVR2A/2B. As a result of it, restoration of each miRNA inhibited both SMAD1/5 and ERK1/2 pathways in Huh7/SNU449 cells and prevented spheroid formation/EMT/stemness/invasion/migration/proliferation/chemoresistance. These miRNAs were sequestered by a single lncRNA, KCNQ1OT1 in the cytoplasm. Hence, deletion of KCNQ1OT1 using CRISPR approach [17] in Huh7/SNU449 cells restored functions of each miRNA and induced chemosensitivity. In addition, shrinkage of tumour size was noted when these knock out cells were implanted to the flanks of NOD/SCID mice suggesting KCNQ1OT1 could be an improved therapeutic target for HCC.

Materials and Methods

Ethical clearance

The Ethics Committee of the Institute of Post-Graduate Medical Education & Research (IPGME&R), Kolkata and the National Centre for Cell Science, Pune had approved the study [Approval ID: Inst/IEC/2015/108; dated 07 July 2015] and [IAEC/2022/B-414] respectively. Written informed consent was obtained from all the participants or legal guardians.

Study Subjects and samples included in the study
Fifty-nine treatment naïve chronic hepatitis patients mono-infected with either HCV or HBV attending the hepatology Clinic of School of Digestive and Liver Diseases, IPGME&R, Kolkata and Indraprastha Apollo Hospital, New Delhi were included in the study and categorized as Chronic Hepatitis B or C (CHB or CHC) (n = 21), Liver Cirrhosis (LC) (n = 17) and HCC (n = 21). Patients co-infected with HEV/HAV/HIV, having co-morbidities like chronic alcoholism, diabetes mellitus or unwilling to enrol in the study were excluded. Normal liver biopsy tissue was obtained from Gall bladder carcinoma patients (n = 11) during cholecystectomy from IPGME&R as routine evaluation of liver metastasis and confirmed after assessment of histology. Both blood and liver tissues were collected for proper disease evaluation and further study.

Details of the study subjects, biochemical, and clinical data is presented in Supplementary Table S1.

Transcriptome profiling

Our small RNA transcriptome profile of liver tissue samples from HCV-HCC vs. normal individuals using Illumina platform has been deposited in the public domain as GSE140370 [18]. After analysis only downregulated miRNAs (log2 fold change ≤ -1.5, P adj≤ 0.05) were considered for this study. Public database GSE21362, GSE40744, GSE74618 were used for validation.

Total RNA isolation and cDNA preparation

About 500ng/2.5µg of RNA was used for cDNA synthesis of miRNA and mRNA using miScript PCR Starter Kit (Qiagen, #218193) or miRCURY LNA miRNA PCR Starter Kit (Qiagen, #339320) and RevertAid reverse transcriptase [ThermoFisher, #EP0441] respectively.

Quantitative Real Time PCR (qRT-PCR)

Both miRNA and mRNA were quantified using PowerUp™ SYBR™ Green PCR master mix (ThermoFisher,) and gene specific primers using QuantStudio 7 Flex RT-PCR machine (ThermoFisher). RNU6B/miR-103a-3p and 18s rRNA were used as an internal controls for miRNA and mRNA respectively. Log2[2−(Ct of gene−Ct of control)]×10^3 was used as fold change in expression of genes.

Bioinformatics analysis

Targets of the miRNAs were analysed using TargetScan (http://www.targetscan.org/vert_80/), mirDB(http://mirdb.org), micro-T-CDS (http://diana.imis.athena-innovation.gr/DianaTools). miRNet (https://www.mirnet.ca/) tool was employed to visualize the overall network of miRNAs. Targets were validated in LIHC and subjected to pathway analysis using KEGG (https://www.genome.jp/kegg/pathway.html).

Lncbase V3.0-DIANA Tools (https://diana.e-ce.uth.gr/Lncbasev3/home) was used to predict the common IncRNA that binds to multiple miRNAs having 8mer binding capacity with score ≥ 0.95.

Cell lines and plasmid information
Huh7/SNU449 cell lines were STR profiled and tested for Mycoplasma. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, HiMedia, #AL111) with 10% FBS (GIBCO, #10082139) in 37°C incubator. Lipofectamine 2000 (ThermoFisher) was used for transfection using manufacturer’s protocol.

Replication competent plasmids of HCV genotype2a, pS52/JFH1 and pSVNeo2/HBV Dimer were gifted by Jens Bukh, Copenhagen University Hospital, Denmark and Prof. Chiaho Shih, University of Texas Medical Branch, Galveston, USA respectively.

Pre-miRNA and 3'UTR sequences of the genes were cloned in pRNAU6.1RNA/Neo vector and psiCHECK™−2 (Promega) vector respectively. The binding regions of the respective miRNA to the lncRNA were cloned into the pGEM-T Easy vector (Promega). pAgo2-Flag (Addgene) and pSpCas9(BB)-2A-Puro (PAX459) (Addgene) [referred as pCas9] were gifted by Edward Chan, and Feng Zhang respectively [19, 20]. All primers and oligonucleotides are listed in Supplementary Table S2.

### 3’UTR Luciferase Assay

Huh7 cells were co-transfected with the 3’UTR-Luciferase construct and control vector/pre-miRNA/pre-miRNA + anti-miRNA oligo independently. Luciferase assay was performed using Dual Luciferase Reporter assay kit (Promega) and Luciferase activity was normalized to the empty vector.

### Immuno-blot analysis

Huh7 cells were transfected with the required plasmids. After 48h, cell extract was prepared using RIPA buffer. About 60–80µg of protein was subjected to polyacrylamide gel electrophoresis, transferred on a PVDF membrane and immuno-blotted with anti-ACVR2A/anti-BMPR1B/anti-CD44/anti-OCT4/anti-NANOG/GAPDH-HRP/anti-Histone-3 (Cell Signalling/Abclonal/Bio-Bharti). Anti-mouse/anti-rabbit-HRP-conjugated secondary antibodies (Santa Cruz) were used as required. Enhanced chemiluminescence (ECL) kit (ThermoFisher) was used to detect specific protein. Detailed antibody list is given in Supplementary Table S3.

### Wound healing assay

Huh7 cells were transfected with the required plasmids. After 24h of transfection, a thin scratch was created at the bottom of the plate and cell migration was monitored using inverted microscope at 0/24/48/72h. The data was analysed using Image J software.

### Cell proliferation assay

Huh7 cells were seeded in a 24-well plate and transfected with the required plasmids. Cells were trypsinized and seeded in a 96-well plate in triplicates. 24h post transfection, tetrazolium salt WST-1 (SigmaAldrich) was added and quantified at 0/24/48/72h in microplate reader.

### Spheroid formation assay
Huh7 cells were transfected with the desired plasmids. 48h post transfection, cells were allowed to grow on ultralow attachment plates (BD, USA) and cultured for 6 days in DMEM-F12 media (Himedia) supplemented with 2% B27 (ThermoFisher) and 20ng/ml of epidermal growth factor (ThermoFisher). The size and number of the tumour-spheres were documented.

Migration and invasion assay

Transfected Huh7 cells were transferred on the upper layer of the Boyden chamber and cell number was counted after staining with crystal violet from the lower part of the membrane at different time points where 30% FBS was used as chemo-attractant. The upper chamber was coated with matrigel for invasion assay.

Biotinylation of KCNQ1OT1 fragments

LncRNA clones were subjected to *in vitro* transcription in a single tube using MEGAscript™ T7 Transcription kit (ThermoFisher). RNA was purified by ethanol precipitation and subjected to biotinylation using the Pierce™ RNA 3’ End desthiobiotinylation kit. RNA was incubated with the extract of Huh7 cells co-transfected with miR-424-5p/miR-223-3p/miR-136-3p/miR-139-5p and ribo-complex was pulled down with streptavidin magnetic beads following manufacturer’s protocol (Pierce™ Magnetic RNA-Protein Pull-Down Kit). RNA was then eluted using Trizol and quantified by qRT-PCR.

RNA Immuno-precipitation (RIP) assay

The plasmid pAgo2-Flag was co-transfected with pCas9/pCas9-KCNQ1OT1 in Huh7 cells. Cells were harvested 48 h post transfection and equal amounts of protein was used for immuno-precipitation with Anti-Flag (Sigma)/Anti-IgG (ThermoFisher) antibody separately, overnight at 4°C. Next day, the RNA-protein complex was precipitated with protein A/G Agarose beads (Sigma) and the RNA was isolated using TRIzol. qRT-PCR was used to quantify RNA.

Mice Experiment

Mice experiments were performed with CRISPR-deleted-KCNQ1OT1-Huh7 cells and vector cells injected subcutaneously into the right dorsal flank of eight weeks old NOD/SCID mice (n = 4). The tumour volumes were measured after 4 weeks using the following formula: \( \pi / 6[(d_1*d_2) \frac{3}{2}] \), where d1 and d2 are two different diameters dimensions of a tumour.

Statistical analysis

Statistical analysis was performed in excel or in the GraphPad prism version 7. All the data were presented as mean ± standard deviation. Unpaired two-tailed Student’s t-test or Mann-Whitney test was done for the data with Gaussian and Skewed distribution respectively. Bonferroni correction (two-way ANOVA) was done for the grouped data analysis. pvalue 0.05 was considered as statistically significant.

Results
Profiling of downregulated miRNAs in the liver tissue of the hepatitis virus infected HCC

The small RNA transcriptome profiling of liver tissue samples from HCV infected HCC patients (n = 5) compared to control individual (n = 5) revealed that miR-424-5p, miR-136-3p, miR-139-5p, miR-223-3p and miR-375-3p were the most downregulated miRNAs in HCC (log₂ fold change ≤ -1.5, P adj ≤ 0.05) as presented in the heatmap (Fig. 1A). The data was also verified using GEO datasets (GSE21362, GSE40744, and GSE74618) between liver tissue of HCC and adjacent normal (Supplementary Table S4).

To understand the molecular mechanism of repression of these tumour suppressor miRNAs in HCC, their expression patterns were determined at different progressive stages of liver diseases from CHC to HCC through LC by qRT-PCR. The results clearly demonstrated that the expression of these miRNAs remain unaltered between normal and CHC, however their expressions were significantly reduced at the end stage liver diseases such as LC (miR-424-5p/miR-223-3p) and HCC (miR-424-5p/miR-136-3p/miR-139-5p/miR-223-3p/miR-375-3p) (Fig. 1B).

Consistent with this data, a similar diminishing trend in the expression of these miRNAs was also observed at different progressive stages of HBV-infected samples such as CHB (miR-424-5p/miR-375-3p), HBV-LC (miR-424-5p/miR-136-3p/miR-375-3p) and HBV-HCC (miR-424-5p/miR-136-3p/miR-139-5p/miR-223-3p/miR-375-3p) compared to normal (Fig. 1C). The data was validated after transfection of pS52/JFH1 and pSV2neoHBV2x in Huh7 and SNU449 cells respectively. (Fig. 1D, 1E).

Identification and validation of pathways targeted by the five downregulated miRNAs in HCC

Next to identify the potential targets of the five downregulated miRNAs, in-silico analysis was performed using TargetScan/miRDB/micro-T-CDS and the expression of each target was verified from TCGA–LIHC datasets. KEGG pathway analysis was employed only with significantly overexpressed target genes and observed that the pathways regulating pluripotency of stem cell signalling was the most enriched pathway (FDR ≥ 3 and p value ≤ 0.05) commonly targeted by these five miRNA independently as presented in Fig. 2A and Supplementary Fig. S1 (A-E) while their target genes were ACVR2A (by miR-424-5p/miR-223-3p), ACVR2B (by miR-424-5p), BMPR1A (by miR-139-5p), BMPR1B (by miR-136-3p and miR-375-3p), all were receptors of BMP signalling pathway [21]. The integrated interaction among these miRNAs and target genes was also verified using miRNet analysis (Fig. 2B).

Next, BMPR1A and ACVR2B were found mostly overexpressed in our small HCC cohort (> 80%) while BMPR1B and ACVR2A were higher in 60% of the samples. All the four genes were upregulated in Huh7/SNU449 cells transfected with either pS52/JFH1 or pSV2neoHBV2x (Fig. 2C & 2D and Supplementary Fig. S1F). The expression of each target gene was diminished significantly upon restoration of the respective pre-miRNAs in Huh7/SNU449 cells independently (Fig. 2E, Supplementary Fig. S1G).
3’UTR-Luciferase assays to confirm binding of miRNAs to the corresponding target gene

Huh7 cells were transfected with either pre-miRNA or anti-miRNA independently along with the respective 3’-UTR-Luciferase reporter constructs. One set was repeated with 3’-UTR-Luciferase constructs having mutation in miR-binding site (MMBS). The reduced luciferase activity in presence of miRNA was restored upon anti-miRNA treatment and in presence of MMBS-Luciferase constructs (Fig. 2F). The binding of each miRNA to the individual target was also verified by immuno-blot analysis with respective antibodies (Fig. 2G). The binding sequences of miRNA and target genes are presented in Supplementary Table S5.

Effect of miRNAs on BMP signalling which induces CSC like features to HCC cells

Next to further evaluate the impact of each miRNA on BMP signalling axis, the level of phospho-SMAD1/5 and phospho-ERK1/2 along with SMAD4 nuclear enrichment were investigated by immunoblot analysis in Huh7/SNU449 cells. It was observed that upon attenuation of either ACVR2A/ACVR2B or BMPR1A/BMPR1B, both pSMAD1/5 and pERK1/2 significantly reduced, as a result nuclear SMAD4 level diminished dramatically compared to mock. Comparable data was observed with anti-sense ACVR2A and BMPR1B treatment in both Huh7/SNU449 cell lines (Fig. 3A-C and Supplementary Fig. S2A, S2B).

So, the expression of downstream EMT markers including Zeb1/Snail/Vimentin/N-cadherin and stem cell markers, CD133/CD44/OCT4/NANOG were determined in Huh7/SNU449 cell lines by qRT-PCR and immunoblot analysis and observed a huge suppression of each of these markers upon restoration of miRNA independently while anti-miRNA treatment showed opposite data (Fig. 3D, 3E and Supplementary Fig. S2C). The expression of the above markers were also reduced upon ACVR2A-AS and BMPR1B-AS treatment in both Huh7/SNU449 cells (Fig. 3F, and Supplementary Fig. S2D).

Keeping similar setting of transfection, the number and sizes of the spheroid formation were noted to be decreased markedly upon restoration of individual miRNA in both Huh7/SNU449 cells than mock, and anti-miRNA treatment (Fig. 3G and Supplementary Fig. S2E) and cells became sensitive to 5FU (Fig. 3I and Supplementary Fig. S2G). Comparable data was obtained when Huh7/SNU449 cells were treated with ACVR2A-AS and BMPR1B-AS (Fig. 3H and Supplementary Fig. S2F).

Impact of miRNAs on cancer cell proliferation, invasion and metastatic potential

The pro-metastatic function of BMP signalling has been reported by many groups [17]. Here, we also observed the proliferation, invasion and migration were reduced drastically upon restoration of individual miRNAs in Huh7/SNU449 cells, while reverse data was obtained as anti-miRNA oligo was treated (Fig. 4A, 4C, 4E and Supplementary Fig. S3A, S3C, S3D, S3G). ACVR2A-AS and BMPR1B-AS treatment in
Huh7/SNU449 cells also showed suppression of proliferation, invasion and migration (Fig. 4B, 4D, 4F and Supplementary Fig. S3B, S3E, S3F, and S3H).

Thus, our observations clearly reinforce that BMP signalling is under surveillance of tumour suppressor miRNAs, downregulation of which triggers CSC like features.

**LncRNA KCNQ1OT1 is the common regulator of all five miRNAs**

To investigate the mechanism of suppression of these beneficiary miRNAs during carcinogenesis, both transcription and post transcriptional regulatory network were explored. Surprisingly, no significant variation was noticed at the pre-miRNA level in the liver tissue of HCC vs. control samples (Supplementary Fig. S4A). Next, “Lncbase V3.0-DIANA Tool” was employed and observed lncRNA-KCNQ1OT1 has binding sites for all five miRNAs. The data was validated using Cytoscape and in our mRNA transcriptome of HCV-HCC samples (Fig. 5A,5B). qRT-PCR analysis depicted that KCNQ1OT1 was abundant in both nuclear and cytoplasmic fractions of Huh7/SNU449 cells and also in our HCC cohort (Supplementary Fig. S4B and Fig. 5C). It was significantly overexpressed upon HCV/HBV infection in two cell lines (Fig. 5D and Supplementary S4C). Poor disease free survival was observed in KCNQ1OT1 high HCC patients (Fig. 5E). The Pearson's correlation plots also depicted a negative association between expression of each miRNA and KCNQ1OT1 in our cohort (Supplementary Fig. S4D).

**LncRNA KCNQ1OT1 sponges miRNAs as an endogenous competitive RNA**

Next, KCNQ1OT1 was silenced in Huh7/SNU449 cells using CRISPR technology (written as KCNQ1OT1-sgRNA), stable cells were selected by the puromycin selection and the expression of each miRNA was observed to be recovered significantly (Fig. 5F and Supplementary Fig. S4E). RIP analysis with anti-Ago2 using wild type and KCNQ1OT1-sgRNA Huh7/SNU449 cells showed enrichment of miRNAs on the RISC of KCNQ1OT1-sgRNA cells compared to control (Fig. 5G, Supplementary Fig. S4F). In vitro interaction study with biotinylated RNA and wild type or KCNQ1OT1-sgRNA Huh7/SNU449 cell lysates revealed miR-424-5p/miR-136-3p/miR-139-5p/miR-223-3p bound to sense lncRNA only while anti-sense RNA was used as negative control (Fig. 5H, Supplementary Fig. S4G). miR-375-3p remains unresolved due to unavailability of binding sequence in the website.

**Role of KCNQ1OT1 in BMP signalling pathway**

Now, the expressions of ACVR2A/ACVR2B/BMPR1A/BMPR1B were quantified by qRT-PCR and immunoblot analysis in KCNQ1OT1-sgRNA-Huh7/SNU449 cells and found significantly lower compared to mock (Fig. 6A and Supplementary Fig. S5A), while combination of anti-miRNAs treatment restored their expression as observed by qRT-PCR (Fig. 6B and Supplementary Fig. S5B). The KCNQ1OT1-sgRNA-Huh7/SNU449 cells also showed less p-SMAD5 and p-ERK1/2 (Fig. 6C, Supplementary Fig. S5C). The level of EMT & stemness markers/spheroid size & number/chemo-
resistance/proliferation/migration/invasion were significantly attenuated in absence of KCNQ1OT1 (Fig. 6D-J, Supplementary Fig. S5D-J). Subcutaneous xenograft tumour model showed massive tumour growth arrest when stable KCNQ1OT1-sgRNA-Huh7 cells were injected to the right dorsal flank of NOD-SCID mice compared to the control vector (Fig. 7A).

Thus, our data suggest that KCNQ1OT1 is overexpressed in HCC and sequesters multiple beneficiary miRNAs, inducing CSC-like features in cancer cells leading to chemoresistance (Fig. 7B). Hence, anti-KCNQ1OT1 treatment has robust therapeutic potential for advanced HCC.

Discussion

In this study, we uncovered the function of the five most downregulated tumour suppressor miRNAs, miR424-3p/miR136-3/miR-139-5p/miR-223-3p/miR-375-3p in HCC and surprisingly observed that these miRNAs convergently inhibited signalling pathways regulating pluripotency of stem cells where targets were type-I and type-II BMP receptors (BMPR1A/BMPR1B and ACVR2A/ACVR2B) which function after tetramerization in presence of BMPs. Restoration of each miRNA independently in Huh7/SNU449 cells arrested CSC-like phenotypes and made cells sensitive to 5FU. Here, we have explored the BMPR1B and ACVR2A receptors for the first time and observed knock down of these two receptors independently also impeded BMP signalling and both Huh7/SNU449 cells lost CSC-like features and cells became sensitive to 5FU. Furthermore, IncRNA-KCNQ1OT1 was identified as a common regulator of all these miRNAs, hence deletion of KCNQ1OT1 in Huh7/SNU449 cells exhibited slow proliferation, reduced stemness & metastatic potential and enhanced chemo-sensitivity. Furthermore, there was visible shrinkage of tumour size in NOD/SCID mice implanted with these cells, suggesting strong potential of KCNQ1OT1 as therapeutic target for advanced HCC.

Our transcriptome data of liver tissue samples from HCV-HCC patients resonate well with the GEO datasets (GSE21362, GSE40744, GSE74618) that reported miR-223-3p/miR-375-3p/miR-424-5p/miR-136-3p/miR-139-5p were among the downregulated miRNAs in both HCV/HBV-HCC. BMP signalling pathway through BMPR1A/BMPR1B and ACVR2A/ACVR2B were commonly inhibited by each of these miRNAs. There is paucity of data related to this signalling. Only a few studies described overexpression of BMP4 protein in HCC liver tissues compared to adjacent non-tumour tissue [22] and low dose BMP4 induces maintenance of CSCs while high dose causes terminal differentiation [13]. Thus the role of BMP4 is little controversial. Wang Xiaotong et al. in 2015 reported that the expression of both BMPR1A/BMPR1B and BMPR2 mRNA are highly variable [23]. Though, we have not verified the level of BMP4, the receptors were mostly overexpressed in our cohort and the two downstream signalling molecules SMAD1/5 and ERK1/2 phosphorylation level was significantly down in presence of each miRNA and also after treatment with BMPR1B-AS/ACVR2A-AS. By recruiting cAMP-responsive element binding protein (CREB) and p300, P-SMAD1/5 induces differentiation of CSCs while p-ERK1/2 triggers both proliferation and differentiation [24, 25]. Hence, the suppression of BMPR1B/ACVR2A also blocked EMT/stemness/chemoresistance and metastasis in HCC cells which is contradictory to the observation of Zhou Changhua et al. in 2018 in
colon cancer progression and metastasis [25]. Further study is required to understand this signalling in HCC.

A few studies have documented the function of IncRNA as competitive RNA for beneficiary miRNAs such as IncRNA-KCNQ1OT1 sponges miR-148-3p, miR-149 to induce oncogenic axis via IGF1R, S1PR1 respectively in HCC [26, 27]. Hence, therapeutic potential of anti-sense IncRNA has been established in many studies e.g., IncRNA ITGB8-AS1 markedly reduced proliferation and tumour growth in colon cancer [28]. In consistence with this, we have identified IncRNA-KCNQ1OT1 which behaved as competitive RNA for all five miRNAs and deletion of KCNQ1OT1 in Huh7/SNU449 cells attenuated CSC like phenotype by arresting EMT/stemness/migration/invasion, and induced chemo-sensitivity suggesting therapeutic potential of anti-KCNQ1OT1 for HCC. Reduced tumour size in NOD/SCID mice injected with the KCNQ1OT1-K/O Huh7 cells corroborated well with this observation.

Therefore, this novel study suggests that as miRNAs are having pleotropic functions, a complete understanding of the regulatory mechanisms of deregulated miRNAs with analogous functions may be essential for successful application of ncRNA based therapy. Since IncRNAs are having power to regulate multiple miRNAs simultaneously, it might be a better therapeutic target to restore the cumulative effect of multiple beneficiary tumour suppressor miRNAs and to prevent cancer.

**Declarations**

**Acknowledgements**

Authors acknowledged all the participants who donated liver tissues. Authors also thank to Multidisciplinary Research Unit of IPGME&R, Kolkata for providing flow cytometry facility. This study was presented in the American Association for the Study of Liver Diseases (AASLD), 2021.

**Conflicts of interest**

All authors disclosed to have no competing interests.

**Availability of data and materials**

The data presented in the manuscript and the materials used for this study may be available upon request to the corresponding author.

**Funding**

The Department of Biotechnology, Government of India has sponsored this study through research grant # [BT/PR12648/MED/30/1897/2017].

**References**


Figure 1

Selection of downregulated miRNAs in HCC liver tissue. (A) Heatmap represents the down regulated miRNAs in liver tissue of HCV-HCC (n=5) vs. Control (n=5). qRT-PCR analysis was performed using (B) & (C) liver tissue samples from progressive disease stages of HCV and HBV infection including chronic hepatitis C (CHC)/ chronic hepatitis B (CHB), liver cirrhosis (LC) and hepatocellular carcinoma (HCC).
respectively along with normal liver; and (D) & (E) Huh7 & SNU449 cells transfected with pS52/JFH1 and pSV2neoHBV2x vs. mock.*,**,***,**** indicate p value <0.05, 0.001, 0.0001 and <0.0001 respectively.

**Figure 2**

Identification of pathway(s) commonly targeted by downregulated miRNAs. (A) Graphical representation of KEGG pathway analysis with validated target genes of five miRNAs. (B) Integrated analysis of the
target genes of five miRNAs using miRNET. Expression analysis of target genes by qRT-PCR using (C) liver tissues of control, HCV and HBV-infected HCC patients, (D) Huh7 cells transfected with pS52/JFH1 and pSV2neoHBV2x for 48 hours and (E) after restoration of each miRNA in Huh7 cells. (F) 3'UTR-Luciferase assay using Huh7 cells co-transfected with wild-type-3'UTR-Luciferase construct, with and without miRNA, and mutant-3'UTR-Luciferase construct + miRNA. (G) Immuno-blots analysis with lysates of Huh7 cells transfected with vector, miRNA and miRNA+anti-miRNA independently and probed with anti-ACVR2A, anti-BMPR1B, anti-BMPR1A, and GAPDH-HRP. *, **, ***, **** indicate p-value <0.05, 0.001, 0.0001 and <0.0001 respectively.

Figure 3

**Impact of five miRNAs on BMP signaling cascade.** Immuno-blots analysis with Huh7 cell lysates (A) transfected with vector (mock) and five miRNAs independently and (B) treated with scrambled oligo, ACVR2A-AS and BMPR1B-AS to verify the expression of total SMAD5, P-SMAD5, ERK1/2, and p-ERK1/2.
respectively. (C) Expression of SMAD4 was verified after separation of nuclear and cytoplasmic fraction of transfected cells. H3 and GAPDH were used as loading control for nuclear and cytoplasmic fraction respectively. qRT-PCR validation of (D) EMT markers and (E) & (F) Stemness markers after restoration of each miRNA and ACVR2A-AS & BMPR1B-AS treatment in Huh7 cells respectively. Huh7 cells were either transfected with vector (mock), miRNA & miRNA+anti-miRNA or scrambled oligo, ACVR2A-AS & BMPR1B-AS and subjected to (G) & (H) Sphere formation assay and (I) & (J) 5-floururacil (125ng/µl) sensitivity assay respectively. *, **, ***, **** indicate p value <0.05, 0.001, 0.0001 and <0.0001 respectively.

Figure 4

Effect of miRNAs on migration and invasion of Huh7 cells. Huh7 cells were transfected with either vector (mock), miRNA, miRNA+anti-miRNA or scrambled oligo, ACVR2A-AS, BMPR1B-AS and subjected to (A) & (B) Cell proliferation assay at 0, 12h, 24h,& 48h; (C) & (D) migration assay using Boyden chamber at
48hrs and (E) & (F) invasion assay with matrigel coated Boyden chamber at 48hrs. *, **, ***, **** suggest p value <0.05, 0.001, 0.0001 and <0.0001 respectively.

Figure 5

LncRNA-KCNQ1OT1 sponges five downregulated miRNAs. (A) Cytoscape analysis to verify interactions among five miRNAs and the predicted long non-coding RNA (lncRNAs). (B) A hierarchical clustering...
analysis with the deregulated lncRNAs in the liver tissue of HCV-HCC (n=5) vs. Control (n=5). Expression analysis of KCNQ1OT1 in (C) liver tissues of control, HCV and HBV-infected HCC, and (D) HCV infected and HBV transfected Huh7 cells. (E) Overall disease-free survival analysis using TCGA-LIHC data. (F) qRT-PCR analysis of miRNAs in CRISPR-deleted-KCNQ1OT1-Huh7 cells. (G) RNA immunoprecipitation assay with Ago2 antibody using mock and KCNQ1OT1 knock out Huh7 cells followed by qRT-PCR analysis. (H) In vitro Biotin-Streptavidin pull down assay with cell extract of mock and KCNQ1OT1 knock out Huh7 cells followed by qRT-PCR analysis of mature miRNAs. *, **, ***, **** indicate p value <0.05, 0.001, 0.0001 and <0.0001 respectively.
Figure 6

Characterization of CRISPR-deleted-KCNQ1OT1 Huh7 cells. qRT-PCR analysis of four target genes (BMPR1A, BMPR1B and ACVR2A, ACVR2B) and immune blot analysis with anti-ACVR2A and BMPR1B using (A) mock and KCNQ1OT1 K/O cells, and (B) mock and KCNQ1OT1K/O cells transfected with five anti-miRNAs. Using Huh7-vector and KCNQ1OT1-K/O cells (C) immunoblot analysis of total SMAD5, p-SMAD5, ERK1/2, p-ERK1/2; (D) qRT-PCR analysis of EMT markers and (E) qRT-PCR and immunoblot
analysis of stemness marker, (F) Tumorsphere formation assay, (G) Chemosensitivity assay using 5 FU and (H) Proliferation assay using WST1 (I) Migration assay using Boyden chamber, (J) Invasion assay using matrigel coated Boyden chamber. *, **, ***, **** indicate p value <0.05, 0.001, 0.0001 and <0.0001 respectively.

**Figure 7**

Mice Xenograft model with KCNQ1OT1 K/O Huh7 cells. (A) NOD-SCID mice was injected with KCNQ1OT1 K/O-Huh7 cells into the right dorsal flank. At different time point, tumour volume and mass were determined. * indicates p-value <0.05. (B) Graphical abstract of the study.
Supplementary Files

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