SNHG9 Promotes the Hepatoblastoma Tumorigenesis via miR-23a-5p/Wnt3a Axis

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Abstract

Background:

Hepatoblastoma is common hepatic tumors occurring children between 0 – 5 years. Accumulating studies has shown lncRNA potential role in distinct cancers progression and development including the hepatoblastoma. SnoRNA host gene 9 (SNHG9) is associated the progression of distinct human cancers but, it's specific molecular mechanisms in hepatoblastoma not unknown.

Methods:

In this study, we estimated SNHG9 expression on hepatoblastoma tissue and cell lines by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). Next, we downregulated and upregulated the SNHG9 expression in hepatoblastoma cell lines and then determined the cell proliferation (CCK-8), colony formation, cellular apoptosis activity. The dual luciferase reporter activity, RNA immunoprecipitation (RIP), biotin RNA pulls down and Spemann's Pearson correlation coefficient assay were performed to establish the interaction between the SNHG9, Wnt3a and miR-23a-5p. Xenograft in-vivo tumorigenicity test was performed to elucidate the role of SNHG9 hepatoblastoma in tumorigenesis. SNHG9 role in Cisplatin drugs resistance in hepatoblastoma was also determined.

Results:

SNHG9 was significantly upregulated in hepatoblastoma tissue and cell lines. SNHG9 overexpression on HUH6 & HepG2 resulted in a significant increase in cell proliferation and clonogenic while SNHG9 knock down resulted in a sustained inhibition of cell proliferation and clonogenic activity. Dual luciferase activity, RNA immunoprecipitation and biotin pull down confirmed the direct interaction of miR-23a-5p with SNHG9. In Xenograft tumorigenicity test showed SNHG9 downregulation significantly reduced the tumor growth on mice. ROC and Kaplan-Meier analysis showed potential prognostic and diagnostic importance of SNHG9 in hepatoblastoma.

Conclusion: We concluded that SNHG9/miR-23a-5p/Wnt3a axis promotes the progression hepatoblastoma tumor.

Background

Hepatoblastoma is the most commonly diagnosed primary embryonic hepatic tumors typically observed in the children's and toddlers between 0 – 5 years and rarely in an adult(1-3). It accounts approximately 1% of all the pediatric malignancies whereas it accounts only 65 – 90% of hepatic malignant tumors(4-6). The annual incidence rate of HB is estimated 1.2 – 1.5 per million of the populations globally(7, 8). The exact cause and pathophysiology of hepatoblastoma is mysterious and is believed to be originated from the immature hepatic precursor cells. Increasing studies has shown their association with numerous genetic diseases/disorders including the Familial adenomatous polyposis (FAP), Beckwith-Wiedemann Syndrome, Trisomy 18(9, 10).

The WNT signaling cascade is an important key regulator of embryogenesis, organ development and homeostasis. However, abnormal Wnt signaling activation and deregulation have been linked to the onset, development and progression of distinct human malignancies (11, 12) And it's one of the important hallmarks of cancer development (13, 14). As a result, further research has to be carried out to understand underlying uncovered molecular mechanisms of WNT signaling in the aggressive development of hepatoblastoma tumors and to identify the new therapeutics targets genes for the treatment of HB patients.

To a date treatment of HB patients mainly relies on surgical resection, neoadjuvant chemotherapy and hepatic transplantation(15-17). Over the last few decades, a revolutionary change has occurred in diagnostic tools, surgical techniques and chemotherapeutics treatments regimes resulting in substantial improvement in survival rate. However, there remains a small group (20 – 25%) of the HB patients with non-resectable HB tumors, chemoresistance and metastasis with a poor survival outcome. As a result, immediate action must be taken to identify and establish new molecular-genetic biomarkers, diagnostic and surgical techniques, and new chemotherapeutics target for the early diagnosis and effective treatment of HB patients.

Non-coding are the heterogenous classes of RNA transcripts including the long noncoding RNA (lncRNAs), microRNAs (miRNAs) and others and which they exert the oncogenic and tumorigenicity function(18, 19). Accumulating studies have showed that noncoding RNA mainly the lncRNAs (>200 nucleotides) and miRNAs (19 -25 nt) have been to be aberrantly expressed in distinct of human malignancies, and are being considered as important emerging key player in cancer paradigm. lncRNA influence the expressions of miRNA targeted genes by interacting with miRNAs and reducing the regulatory effects of mRNAs(20, 21). For examples lncRNA UCA1 sponges with miR-240-5p to promote glioma via upregulation of ZEB1. Similarly, in papillary thyroid carcinoma lncRNA Gas5 act controls the PTEN expression by sponging miR-222-3p (22, 23). Small nucleolar RNA host gene 9 (SNHG9) 551 nucleotide base pair intergenic lncRNA located in chr16p13.3 have all been linked to the onset and progression of distinct of cancers including pancreatic cancer [21], glioblastoma [22], lungs cancer [23], ovarian cancer [24]. However, exact function and underlying mechanism of SNHG9 in hepatoblastoma tumorigenesis is unknown and need to be explored out.

Similarly, increasing evidences have shown that deregulated miRNA is associated with cancer progression(24, 25). For an instance miR-21 promotes hepatocellular carcinoma(26), miR-34s upregulated in hepatoblastoma (27), miR-675 upregulated in osteosarcoma (28). Previous researchers have shown the dysregulation of miR-23a-5p in distinct cancer including pancreatic ductal adenocarcinoma (downregulation), glioblastoma and esophageal carcinoma(29-32). Nevertheless, the function of miR-23a-5p in hepatoblastoma tumor progression is undiscovered and need to explore out.

In current study, we investigated the expression level, specific functional and underlying molecular mechanisms of SNHG9 and miR-23a-5p. Our study findings, demonstrate that SNHG9 is significantly upregulated in hepatoblastoma tissue and is closely associated with poor prognosis in hepatoblastoma. SNHG9 promotes the hepatoblastoma tumorigenesis through wnt3a/miR-23a-5p axis. Hence, SNHG9/miR-23a-5p/wnt3a might be novel promising therapeutics targets for hepatoblastoma patients.

Material And Methods

Human Clinical specimen:

Between 2016 and 2020, a total 40 pairs of the hepatoblastoma tissue including the adjacent matching normal hepatic tissue were obtained from pediatric patients who underwent hepatic surgery in Shanghai Tenth Peoples Hospital, China in between. **Table 1 shows the** clinicopathological characteristics of individual patients in detail. This study was sanctioned by the Institutional Research Ethical Review Committee. None of the patients undertaken in this study had never received the radiotherapy and chemotherapy. A verbal and written consents were obtained from all the pediatric patient's parents prior to collection of human clinical sample for research.

ATCC Hepatoblastoma cell lines (HUH6 & HepG2) and normal hepatic cell line (QSG7701) were obtained from the Shanghai Chinese Academy of Cell Collection. HUH6, HepG2 and QSG-7701 cells were cultured DMEM, Minimum Essential Medium (MEM) and RPMI 1640 respectively, incorporated with 10% FBS and 100U/ml Penicillin G/Streptomycin. These cell lines were incubated at 37⁰c in 5% CO₂ incubator. Culture medium (DMEM, MEM & RPMI) and FBS were purchased from Gibco (Grand Island, NY, USA).

Hepatoblastoma (HUH6 and HepG2) cell lines transfections:

For transient hepatoblastoma cell lines transfection three candidates of si-SNHG9 were chemically synthesized by Gene Pharma (Shanghai). Meanwhile, for the stable transfection pLv-SNHG9-shRNA was synthesized from Keli Biotechnology (Shanghai, China). In addition miR23a-5p mimic, miR23a-5p inhibitor and matched negative controls (miR-NC) were synthesized by Shanghai Gene Pharma Inc (Shanghai China). HB cell transfection was carried out according to manufactured instructions. Briefly, the 2.5 x 10⁵ hepatoblastoma cell (HUH6 & HepG2) cells are seeded on 6-well plate and incubated for 18 – 24 h at 37⁰c to allow 30 – 40% confluency growth. On the 2nd day old cultured medium from 6 well plate was pipetted out and washed with 1x ice cold PBS. Next, 1000 µl of Serum free Opti-MEM is added to each well. 200µl of siRNAs/miRNA mimics/inhibitors-Lipofectamine 2000 mixture was prepared by adding 2µg (6µl 2 OD) of siRNA/miRNA mimics/inhibitors and 4µl of Lipofectamine-2000 in 200µl of serum free OPTI-MEM medium and to respective 6-well plate and was incubated 4 – 6 h 37⁰c. After 4 – 6 h OPTI-MEM medium was substituted with DMEM/MEM medium and incubated at 37⁰c for 48 – 72 h. The knockdown efficacy of siRNA/miRNA mimics/inhibitors was validated by performing the qRT-PCR from the total mRNA extracted from transfected HB cell lines. The sequences of SNHG9 siRNA, hsa-miR-23a-5p/mimics/inhibitors are enlisted in **Supplementary file1**.

Total mRNA isolation and Quantitative real-time PCR (qRT-PCR):

Total mRNA was isolated from the hepatoblastoma tissues and cell lines using the Trizol reagent (Invitrogen), as per the manufacturer`s guidelines. The Prime Script RT reagent kit (Takara, Dalian, P.R China) is then used to reverse-transcribe the mRNA into cDNA. Meanwhile, for the microRNAs, mRNA was reversed transcribed in cDNA using miRNA-specific loop RT primer synthesized by Ribobio (Guangzhou, China). cDNA of specific target gene (SNHG9, Wnt3a c-MYC, β-catenin and miR23a-5p) was amplified using SYBR Premix Ex Taq II (Takara Biotechnology, China) on qRT-PCR ABI Prism 7500 machine (Applied Biosystems, Thermo scientific). GAPDH and U6 are used as the internal control. Differential expression of target genes was calculated by 2-ΔΔCT method. The primers sequence of the target genes is enlisted in **supplementary file 1**.

Proteins extraction and Western SDS-PAGE electrophoresis:

Protein from the stably/transiently knocked from hepatoblastoma cell lines (HUH6 & HepG2 cells) was extracted using the RIPA lysis buffer (Biyuntin, China) with the protease inhibitor (PI) cocktail (Cell Signaling Technology, USA) and phenylmethanesulfonyl fluoride (PMSF) (Biyuntin, China). The BCA kit (Biyuntin, China) was used to determine the protein concentration and denatured at 100⁰c for 10 mins. 40 – 80 µg denatured total protein sample was separated on 10 -12% SDS-PAGE. Separated protein were blotted into the nitrocellulose membrane. Nitrocellulose membrane were then transferred to 5%BSA blocking solution for 60 min and subsequently the nitrocellulose membrane was incubated with specific primary antibodies Wnt3a (ab2194120, β-catenin (ab32572), β-Actin(ab170325) (1:5000), C-Myc (ab32072), Survivin (ab76424), Blc-2 (ab182858), Bax (ab32503), Cleaved

2324) at 4⁰c for overnight.

Following the overnight incubation, the nitrocellulose membrane was rinsed four times with PBST and then incubated with secondary, HRP- conjugated goat anti-rabbit antibody (ab6721) at room temperature for 90 -120 mins. Next, nitrocellulose membrane is treated with ECL western blotting substrates and the chemiluminescence's signal generated from nitrocellulose membrane was detected using AmershamTM A600 chemiluminescence film scanner (GE Healthcare Life Sciences). Beta actin was used as internal control for the validation of protein loading samples. All the antibodies related to gene of interest were purchased from Abcam.

Cell proliferation test:

Cell proliferation activity of hepatoblastoma cell (HUH6 & HepG2) after the subsequent transfection with SNHG9 siRNA/miR23a-5pmimics/inhibitors/SNHG9 OE plasmid was assed by CCK-8 assay (CCK-8, Biyuntian, China) assay. Hepatoblastoma cell lines (HUH6 & HepG2 cell) transfected with si/shRNA/miRNA mimics/inhibitors were seeded t a density of 1×10^3 cells/well in 96 wells plate and incubated humidified 5% CO_2 incubator at 37°C for 5 days. 10 μl of CCK8 solution (Biyuntian, China) was added to each well and the absorbance of the colorimetric reaction of successive 5 days was determined using BioTek multi-mode microplate reader (BioTek, USA). Cell proliferation activity was normalized with zero hours' time absorbance. Cell viability was calculated and plotted using Graph Prism. Each experiment was repeated thrice.

Clonogenic Assay:

Transfected hepatoblastoma (HUH6 & HepG2) cells were plated at the density of 1×10^3 per wells into a 6-well plate and incubated for 14 days at 37°C . Briefly, following the 14 days incubation, the culture medium from the 6-well plates pipetted out and washed with 1x PBS. In 6 well plate cell colonies were fixed with 4% paraformaldehyde, then wash with PBS before staining with 0.05% crystal violet. Eventually, the crystal violet from 6 well plate is removed and washed with tap water and allow the plate to dry. Using the Image J software, the number of colonies in each well were counted and presented in bar chart using the Graph prism.

Flow Cytometric analysis for Apoptosis Assay:

To determine the Cellular apoptosis activity of transfected hepatoblastoma (HUH6 & HePG2) cell we utilized the Annexin V fluorescein isothiocyanate (FITC)/ propidium iodide double staining Apoptosis Detection Kit. After the trypsinization, transfected HB cells were collected in tubes and centrifuged at 1000 RPM for 5 mins. The cells pellet collected on bottom of tube was washed twice with ice cold 1x PBs before being suspended in Annexin binding buffer. The cell suspension was then distributed distinct tube at the density of 1×10^5 cells/tube followed by the double staining solution. Initially, cells were stained with Annexin-V FITC for 15 minutes and then, with propidium iodide (PI) for 5 minutes. Eventually, flow cytometry (BD Biosciences company, USA) was utilized to detect the apoptotic cell. The Flow Jo Software was used to calculate the percentage of the cellular apoptosis. Each experiment was performed in triplicate.

Isolation of Cytoplasmic and Nuclear RNA:

The Ambion PARIS Kits (Invitrogen, NY, USA) has been used for the isolation of cytoplasmic and nuclear fractional RNA from hepatoblastoma mammalian cell lines (HUH6 & HepG2). The relative concentration/fractional distribution of SNHG9, U6, 18s and GAPDH was calculated based on qRT-PCR findings. U6, 18s and GAPDH were used as nuclear and cytoplasmic control transcript.

RNA Immunoprecipitation Assay (RIP) Assay:

The EZMagna RNA-binding protein immunoprecipitation kit (Millipore, MA, USA) was utilized to validate the interaction between the SNHG9 and miR23a-5p and was performed in accordance with manufactured guidelines. In brief, pCDNA-SNHG9 or miR23a-5p mimics transfected HUH6 and HepG2 cells were plated in 6 well plate and incubated 48 h. After 48 h of transfection, HB cell lysate was obtained after the subsequent treatment of HUH6 and HepG2 cell with RIP lysis buffer. Magnetic beads coated with Ago2 antibody (Millipore's, USA) or anti rabbit IgG (Millipore's, USA) antibody mixed with cell lysate buffers and incubated for 6 h at 4⁰c. After the 6 h immunoprecipitated RNA was extracted with the subsequent elution of protein beads. Eventually, qRT-PCR was performed to analyze the extracted precipitated RNA.

Biotin Pulldown Assay:

Biotin label antisense and sense SNHG9 RNA and DNA probes has designed, synthesized and purchased from Sangon Biotech (Shanghai, China). Hepatoblastoma (HUH6) cell lysate were mixed with the biotinylated SNHG9 RNA/ DNA probes and was incubated approximately at 25⁰c for 1h. The streptavidin-agarose beads (Invitrogen) were mixed to mixture to elute the biotin-coupled RNA complexes. Eventually, qRT-PCR was performed to assess the abundance of SNHG9 and has-miR23a-5p in pull-down materials.

Xenograft Tumors:

The vivo animal tumorigenicity experiment was performed to validate the oncogenic potential of SNHG9. 4 weeks old BALB/c nude mice of was used for this experiment. BALB/c nude mice were purchased and randomly classified into two major groups Lvsh-NC and lv-shSNHG9. Each group consisting of 6 BLAB/C nude mice. For the tumorigenicity assay, lvsh-NC and lv-shSNHG9 transfected HUH6 were collected, centrifuged and resuspended in ice cool 1X PBS. And then, 5x10⁶ of lv-shNC and lv-shSNHG9 transfected HUH6 were injected subcutaneously into the posterior flank of BALB/c nude mice. After six days of injection of HUH6 cell suspension in mice the tumors growth on mice was observed and evaluated in every three days. The volume of the tumors was measured using an equation $V=0.5 \times D \times d^2$ where V= volume, D is the longitudinal diameter and d is latitudinal diameter of tumors). The mice were killed by cervical dislocations after 21 days and the weight of tumors and volume of tumor is measured. All the animal experiment was carried out in compliance to the NIH guidelines for the care and use of laboratory animals.

2.7.1 Bioinformatics analysis:

Similarly, we utilized the RegRNA 2.0 (<http://regrna2.mbc.nctu.edu.tw/detection.html>) database to identify the possible miRNA binding to SNHG9. Similarly, we utilized the RNA hybrid database to possible binding site of SNHG9 to miR-23a-5p. Similarly, we utilized the Targets (http://www.targetscan.org/vert_72) can database to identify the miR-23a-5p targeting genes. Similarly, we utilized the Starbase (<http://starbase.sysu.edu.cn/>) and RNAhybrid (<https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid>) to identify the binding sites of miR-23a-5p in Wnt3a target gene.

Dual Luciferase Assay:

The putative target binding site of miR23a-5p on SNHG9 and Wnt3a wild and mutant variants were cloned into pmirGLO(PsiCheck2) firefly luciferase vectors (Promega, Madison WI, USA). HUH6 and HepG2 cells were seeded at

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the density of 1×10^5 cells in 12 well plate and incubated at 37°C for 24 h. SNHG9 {SNHG9-Wt & SNHG9-Mut} and Wnt3a [Wnt3a-WT & Wnt3a-Mut] constructed vector was co-transfected into HepG2 and HUH6 cells with miR23a-5p mimics/inhibitors using the Lipofectamine 2000 (Invitrogen, USA). Forty-eight hours after the post transfection, the HepG2 and HUH6 cell was lysed using passive lysis buffer. The cell lysate was then collected and centrifuged. And eventually used to measured luciferase activity. Luciferase activity was determined using the Dual-Luciferase Reporter Assay system (Promega, China).

Statistical analysis:

All the finding of this study was presented in mean $\pm$ SD. Statistical analysis was conducted using SPSS version 16.0 (IBM, NY, USA) and GraphPad prism version 8.0 (GraphPad Software, La Jolla, CA). Student *t*-test and *one-way ANOVA* was used $\rightarrow$ calculate and evaluate the two and more groups. Spearman

s rank correlation coefficient test was used to determine the correlation between two groups. Study finding with p value ≤ 0.05 was defined as statistically significance.

Results

SNHG9 is Upregulated in Hepatoblastoma tumors and Cell lines

SNHG9 expression was found significantly elevated in hepatoblastoma tissue than in adjacent normal hepatic tissue (**Figure 1A**). Similarly, SNHG9 expression on hepatoblastoma cell lines (HUH6 & HepG2) is remarkably high compared normal hepatic cell lines (QSG7701) (**Figure 1B**). Similarly, the impact on the relative expression of SNHG9 on subsequent SNHG9 overexpression and knockdown on the hepatoblastoma cell lines was also studied. As shown **in Figure 1C**, a significant high expression SNHG9 was noted in hepatoblastoma cell lines (HUH6 & HepG2) transfected with SNHG9 overexpression plasmid. Meanwhile, in SNHG9 siRNA Knockdown hepatoblastoma cell lines (HUH6 & HepG2) there was a significant reduction in the SNHG9 expression (**Figure 1D**). Next, NE-PER nuclear and cytoplasmic extraction kit was then used to determine the subcellular distribution of the SNHG9, both the hepatoblastoma cell lines (HUH6 & HepG2) and found SNHG9 predominately located in the cytoplasm like that of majority lncRNA (**Figure 1E and 1F**). Based on the above findings, we confirmed that SNHG9 is highly overexpressed in the hepatoblastoma tissue and cell lines suggesting that and it may promote hepatoblastoma tumorigenesis.

SNHG9 expression correlation with clinicopathological factors and it's prognostic and diagnostic importance Hepatoblastoma patients:

SNHG9 expression among hepatoblastoma tumor patient was significantly upregulated (**Figure 1A**). Next, we performed the Chi-square test to evaluate the association in between SNHG9 expression and patient's clinicopathological characteristics, and noted that SNHG9 expression is not correlated to gender, age, tumor size, TNM staging. However, SNHG9 expression is positively correlated with histology of HB tumor ($p=0.056$) (**Table 1**). In additional, Kaplan-Meier survival analysis has been used to evaluated the influence of SNHG9 expression in the overall survival (OS) rate of HB patients and found patients with high SNHG9 expression had recurrent recurrences and low 5-year survival rate ($p=0.0161$) then patients with low SNHG9 expression (**Figure 2A**). In addition, to ascertain the diagnostic value of SNHG9, a ROC curve analysis was performed which revealed a high degree of sensitivity and specificity ($\text{AUC}=0.8928$; $p\text{-value}<0.0001$) (**Fig 2B**). Taking in consideration of above findings,

SNHG9 may serve as an independent predictor of overall survival. And it could be a potential promising novel biomarker for the prompt early diagnosis and prognosis of HB patients.

SNHG9 expression level impacts on cellular proliferation, colonization and apoptosis activity in Hepatoblastoma tumorigenesis.

To investigate how SNHG9 expression variation influences on the biological activity of HB cell we determine the cell proliferation, colony formation and cellular apoptosis activity on SNHG9 siRNA knockdown and overexpressed HepG2 and HUH6 cell. **(Figure 3A-F)**. HB cell lines transfected SNHG9 overexpression plasmid showed a significantly high level of cell proliferation (CCK8) and clonogenic activity **(Figure 3A and 3B)**; however, SNHG9 siRNA knockdown HB cell lines showed a remarkable depletion in cell proliferative and clonogenic abilities **(Figure 3C & 3D)**.

In addition, flow cytometry analysis has been performed to investigate the effect of SNHG9 overexpression and knockdown on cellular apoptosis. SNHG9 siRNA knockdown HUH6 and HepG2 cell had a significantly high cellular apoptosis **(Figure 3E)** while, SNHG9 overexpressed HUH6 & HepG2 cells showed a remarkable reduction in cellular apoptosis **(Figure 3F)**. In addition, SDS-PAGE electrophoresis was used to investigate the effect of SNHG9 depletion and overexpression on apoptosis related proteins (Bcl and Bax) and to validate the flow cytometer apoptosis findings. SNHG9 overexpression in HB cell resulting in a substantial increase in BCL expression level and remarkable reduction of BAX protein **(Figure 3H)**. Meanwhile, in SNHG9 knockdown on HB cell lines showed a significant reduction in the BCL and a significant increase in BAX protein level **(Figure 3G)**. Based on above findings, we established that SNHG9 enhances the cellular proliferation and supports the growth and progression of HB tumors.

SNHG9 promotes the HB tumorigenesis through Wnt/ β -catenin Pathway in Hepatoblastoma tumors

To investigate the role of SNHG9 in the deregulation of **Wnt/ β -catenin** signaling pathways during the hepatoblastoma tumorigenesis. Initially, we investigated the effect of SNHG9 depletion and overexpression on the differential expression levels of Wnt/ β -catenin pathway associated genes and proteins including the Wnt3a, β -catenin, c-Myc and survivin. SNHG9 siRNA knockdown on the HUH6 & HepG2 cell resulting in a significant reduction in Wnt3a and other target genes (β -catenin, c-Myc and survivin) **(Figure 4A & 4B)**. In the meantime, SNHG9 overexpressed on HUH6 & HepG2 cells resulting in remarkable increase in the Wnt3a, β -catenin, c-Myc and survivin mRNA expression **(Figure 4C and 4D)**. In addition, we used SDS-PAGE electrophoresis to demonstrate SNHG9 knockdown and overexpression effects on the relative Wnt3a, β -catenin, c-Myc1 and survivin protein expression. A significant reduction in Wnt3a, β -catenin, c-Myc1 and survivin protein expression was reported on SNHG9 knockdown HUH6 & HepG2 cell lines **(Figure 4E)**. Meanwhile, a remarkably increase in Wnt3a, β -catenin, c-Myc and survivin protein expression was reported on SNHG9 overexpressed HUH6 & HepG2 cell lines **(Figure 4F)**. These findings confirmed that SNHG9 utilizes the Wnt3a/ β -catenin signaling pathway for promoting hepatoblastoma tumorigenesis.

miR-23a-5p deregulation promotes the hepatoblastoma tumorigenesis:

miR-23a-5p is the potential tumor suppressor in distinct cancers. As shown in **Figures 5A and 5B** miR-23a-5p expression level is significantly low on hepatoblastoma tissue/cell lines (HUH6 & HepG2) in contrast to normal liver tissue/cell lines (QSG7701). Similarly, we determine the miR-23a-5p expression and biological activity of HUH6 and HepG2 cell lines. miR-23a-5p is a potential tumor suppressor and inhibitors. A significantly high expression of miR-23a-5p

expression was observed in miRNA-23a-5p mimics transfected HB cell line compared to negative control (**Figure 5C**). Meanwhile, HB cell transfected with miR-23a-5p inhibitors showed a substantial low miR-23a-5p expression compared to negative control (**Figure 5D**). Next, miR-23a-5p mimics transfection on Hb cell lines resulting in a severe reduction on cell proliferation and colony formation activity (**Figure 5E and 5F**). Meanwhile, a significant enhanced cell proliferation and clonogenic activity was reported in miR-23a-5p inhibitors transfected HB cell lines (**Figure 5G and 5H**). These above findings clearly suggested miR-23a-5p deregulation promotes the hepatoblastoma tumorigenesis.

SNHG9 directly interact with has-miR-23a-5p and negatively regulates it activity.

Accumulating studies have shown that lncRNAs acts as ceRNA or molecular sponges to particular miRNAs to influence the biological activity of them. We used RNA hybrid online database was used to elucidate possible binding SNHG9 and miR23a-5p. SNHG9 has potential putative binding sites for miR23a-5p (**Figure 6A**). Dual luciferase assay was used to establish the interaction in between the SNHG9 and miR-23a-5p. A significant reduction in luciferase activity was reported in HB cell lines co-transfected with SNHG9-WT (Wild) reporter vector and miRNA23a-5p mimics compared the HB cell lines co-transfected with SNHG9-Mut (Mutant) vector and miR-23a-5p mimics. (**Figure 6B and 6C**).

Likewise, RIP assay reveled that SNHG9 and miR-23a-5p expression was remarkably high in Ago2 containing beads than in IgG group (**Figure 6D & 6E**), suggesting strong interaction between miR23a-5p and SNHG9. Similarly, Biotin pulldown has also been used to confirm the interaction between SNHG9 and miR23a-5p. Biotin pull down assay showed that relative SNHG9 and miR-23a-5p expression is relatively high in anti-sense DNA probes compared to sense DNA probes (**Figure 6F & 6G**). Similarly, we investigated the impact of SNHG9 knockdown and overexpression SNHG9 on miR-23a-5p expression in HepG2 and HUH6 cell. As shown in **Figure 5I**, a significant increase in has-miR-23a-5p expression level reported in siSNHG9 knockdown HB cell lines. Meanwhile, a significant reduction in miR-23a-5p expression was observed in SNHG9 overexpressed hepatoblastoma cell lines (**Figure 5H**). Furthermore, correlation analysis was used to explore the association between SNHG9 and miR23a-5p hepatoblastoma tumorigenesis, and the findings showed that SNHG9 was is negatively correlated with miR23a-5p (**Figure 5J**). All of these experimental findings indicate that SNHG9 interacts directly to miR-23a-5p and negatively modulates it`s functions.

miR-23a-5p downregulates Wnt3a and Hepatoblastoma tumorigenesis:

miR-23a-5p expression was significantly low in hepatoblastoma tissue and cell lines (**Figure 5A and 5B**). Wnt3a expression in hepatoblastoma tissue was found significantly upregulated (**Figure 6A**). And then, the impact of subsequent transfection of HUH6 and HepG2 cell with miR23a-5p mimics and inhibitors on the relative expression of Wnt3a mRNA and protein was determined. In HB cell lines transfected with miR-23a-5p mimics there was significant reduction on Wnt3a mRNA and protein expression. Meanwhile, miR-23a-5p inhibitors transfected HB cell lines showed a significant enhancement in Wnt3a mRNA and proteins expression (**Figure 6B and 6D**). Similarly, the impact on the expression level of Wnt3a mRNA and proteins on the subsequent

Next, we performed the dual luciferase activity to investigate the interaction in between Wnt3a and miR23a-5p. The predictive bindings sites of miR-23a-5p on Wnt3a was identified using RNA hybrid online database (**Figure 6J**). A decreased luciferase activity was reported in hepatoblastoma cell lines co-transfected with Wnt3a Wild types of reporter and miR-23a-5p mimics whereas increased luciferase activity was in HB cell lines observed in

Wnt3a Wild/mutant vector and with miR23a-5p inhibitors (**Figure 6G and**

6H). Similarly, Spearman's correlation analysis was done to elucidate the correlation in between Wnt3a and miR23a-5p and was found negatively correlated (**Figure 6I**). However, Wnt3a and SNHG9 is positively correlated (**Figure 6J**). These above findings indicate miR23a-5p is tumor suppressor and downregulate the Wnt3a expression in hepatoblastoma tumorigenesis.

Has-miR-23a-5p/Wnt3a showed its involvement in the activity/role of SNHG9 in Hepatoblastoma tumor progression:

Wnt3a mRNA and protein expression was found significantly increased in HepG2 and HUH6 cell transfected SNHG9 overexpression plasmids however; a significant attenuation in Wnt3a protein and mRNA expression was observed in miR-23a-5p and sh-SNHG9 overexpression plasmid co-transfected HepG2 and HUH6 cell (**Figure 6C and 6E**). Thus, these finding clearly suggest the involvement of has-miR-23a-5p/Wnt3a in SNHG9 activity for the progression of hepatoblastoma tumors.

SNHG9 Promotes Hepatoblastoma tumor progression in vivo

The xenograft tumorigenicity test was performed to elucidate the oncogenic potency of SNHG9 in vivo. We performed the qRT-PCR to confirmed the knockdown efficacy of the sh-SNHG9 before the subcutaneous injection in mice and found significantly low SNHG9 expression in HUH6 transfected with sh-SNHG9 compared to control (shNC) (**Figure 7A**). After the confirmation of knockdown efficacy, we inoculated the stably sh-SNHG9 and NC-SNHG9 knockout HUH6 cell suspension subcutaneously on the posterior flank of 4 weeks BALB/c nude mice. In vivo tumorigenicity showed, a remarkable reduction in both the tumor size ($p < 0.001$) and weight ($p < 0.0001$) among nude mice injected with stably knocked down sh-SNHG9 HUH6 cells compared to negative control group of mice (**Figure 7B, 7C and 7D**). Further, we performed the SDS PAGE electrophoresis to observed the effect on the expression of Wnt3a/ β -catenin related pathway proteins extracted from the stable knocked down Nc-SNHG9 and sh-SNHG9. WB analysis showed found significant reduction in β -catenin, c-MYC, Wnt3a, survivin protein expression level in protein extracted from stable (sh-SNHG9) knockdown mice in contrast proteins extracted form tumors developed in Nc-SNHG9 injected nude mice (**Figure 7E**). Collectively, based on above experimental findings confirmed SNHG9 is oncogenic and promotes hepatoblastoma tumorigenesis.

SNHG9 Contributes the Cisplatin chemoresistance in HB cell:

To examine the role of SNHG9 in cisplatin chemoresistance in HB, we initially we determine the ICD (inhibitory concentration dose) of the cisplatin on hepatoblastoma cell lines (HUH6 & HepG2 cell) and found ICD for HepG2 (12 μ M) and 20 μ M for HUH6. Next we performed CCK8 to determine the cell viability percentage after treatment with cisplatin. SNHG9 siRNA knockdown cell after the subsequent treatment with IC50 cisplatin showed a significant decrease in cell viability compared to negative control group. However, the IC50 cisplatin treated SNHG9 overexpressed HB cell lines showed higher cell viability compared to control groups (**Figure 8A and 8B**). Similarly, we determined the cellular apoptosis on DMSO and Cisplatin treated cell and noted a reduced in cellular activity in DMSO treated cell compared to Cisplatin treated cell (**Figure 8C and 8D**). The cellular apoptosis rate in HUH6/HepG2 cells knockout with siSNHG9 showed remarkably high apoptosis activity compared to negative control. Meanwhile, HUH6/HepG2 cell lines transfected SNHG9 overexpression plasmid showed a lower apoptosis activity compared to negative controls. In additional, we examined the cleaved caspase-3 and 9 relative protein expression in DMSO and Cisplatin treated hepatoblastoma cell lines and found that DMSO treated HB cell lines had significantly low level of in cleaved caspase-3, and 9 protein expression than the cisplatin treated HB cell lines (**Figure 8E& 8F**). This finding

chemoresistance. As shown in (**Figure 8G-8I**) significant reduction in

tumor, tumor weight and tumor volume in lv-shSNHG9 cisplatin injected mice in comparison to lv-shSNHG9 DMSO. Thus, we can confirm the SNHG9 contributes in cisplatin chemoresistance development.

Discussion

Hepatoblastoma is the common hepatic malignancy tumor in children with a poor prognosis(1). The disease is slowly progressive thus, clinicopathological examination is not adequate for the early prompt diagnosis of the disease. A significant improvement in the diagnosis, treatment and management of the patients was achieved with the identification of the novel biomarkers serum alpha feto protein (sAFP). However, sAFP is not highly precise and reliable in diagnosis of tumors at early stage. Thus, new novel therapeutics biomarkers must be identified for the early diagnosis and effective treatment of patients(33). Accumulating studies has reported LncRNAs, a noncoding RNA (lncRNA) abnormally upregulated in distinct cancers and may have significant role in the development and progression of distinct human cancers including the colorectal, breast, hepatocellular, pancreatic, glioblastoma via regulating the distinct signaling pathway(34-39). Similarly, accumulating studies has showed abnormally upregulated distinct lncRNAs in hepatoblastoma. **Dong. R et al** on wide genomic analysis identified 2736 differently expressed lncRNAs, 1757 of which were up-regulated and 979 were downregulated in hepatoblastoma tissue(40). Previous studies have proven that SNHG9 acts oncogenes for the development and progression of distinct human cancers including pancreatic(31), glioblastoma(32), non-small lungs cancer (NSCLC)(30), bladder cancer(41), prostate(35). However, the biological function and underlying molecular mechanisms of SNHG9 in hepatoblastoma tumor progression is unknown and need to elucidate.

In the current study, we elucidate the critical role of SNHG9 in hepatoblastoma progression and underlying mechanisms. SNHG9 was conspicuously upregulated in the hepatoblastoma tissue and HB cell lines. In additional, our study findings showed that SNHG9 overexpression is closely related to advance stage of disease and has a poor survival outcome. In addition, to elucidate the biological role of SNHG9 we knockdown and overexpressed the SNHG9 in HB cell lines and performed CCK-8 and clonogenic assay. SNHG9 knock-out resulting in significant reduction in the cell proliferation and clonogenic activities however, SNHG9 overexpression on HB cell enhances the cell proliferation and clonogenic activity thus confirmed it's involvement pathophysiology of hepatoblastoma tumorigenesis. We then investigated fractional distribution of the SNHG9 in cell and found mainly concentrated in cytoplasm. Accumulating studies have shown that cytoplasmic lncRNAs, competes with the microRNAs by acting as ceRNAs and influences miRNAs inhibitory activity on the target genes and modulating cancer progression. We assumed that SNHG9 acts as ceRNAs in hepatoblastoma. For the confirmation using the online database we identify the possible miRNA interacting with SNHG9 and also miR-23a-5p. The possible binding sites of miR-23a-5p on SNHG9 was identified using the RNA hybrid online bioinformatics software. qRT-PCR finding showed reduced miR-23a-5p expression in hepatoblastoma tissue compared to normal hepatic tissue. In additional, a several studies has shown tumor suppressor nature in pancreatic cancer, hepatoma and so on(29, 42). However, the role of miR-23a-5p in hepatoblastoma tumors is. Similarly, a reduced CCK-8 and colony formation activity was reported on HB cell lines transfected with miR-23a-5p mimics. A reduced dual luciferase activity was reported in HB cell lines co-transfected with SNHG9 WT vector and miR-23a-5p mimics. Similarly, increased miR-23a-5p expression was reported in SNHG9 siRNA transfected HB cell lines. The Spearman's Pearson correlation analysis showed a negative correlation in between SNHG9 and hsa-miR-23a-5p. Based on the above findings of our study we confirmed that SNHG9 directly interact with miR-23a-5p and interferes in miRNAs activity.

The canonical Wnt signaling pathway participate in normal functioning of diverse physiological processes

Loading [MathJax]/jax/output/CommonHTML/jax.js resulting in development and progression of cancer(1, 13, 43, 44). We

identified miR23a-5p binding sites on Wnt3a using RNA hybrid database and showed reduced luciferase activity on HB cell lines co-transfected with Wnt3aWT and miR-23a-mimics. Similarly, western blot findings and qRT-PCR finding also showed decreased Wnt3a expression in miRNA mimics transfected cell. Spearman's correlation test showed negative correlation between Wnt3a and miR23a-5p. All these findings provide a strong evidence that SNHG9 promotes hepatoblastoma tumorigenesis via downregulating the miR-23a-5p and upregulation of Wnt3a.

HB is malignant tumor occurring in children. Currently, treatment of the disease mainly depends upon the surgical resection, chemotherapy and neoadjuvant chemotherapy. However, the chemotherapy response in post-operative patients progressively decreased on repeated chemotherapy leading to failure in treatment and death of patients. In recent years, many researchers have demonstrated that lncRNAs contribute in development of chemoresistance. Zhang et al (2018), reported lncRNA KcNQ10t1 confers the cisplatin resistance in tongue cancer via miR-211-5p mediated Ezrin/Fak/Src signaling(45). Li et al reported that lncRNA UCA1 contributes in cisplatin resistance in ovarian cancer with the regulation of miR-143/FOSL2 pathway(22). Dai et al (2020) has shown lncMALT1 regulates the Cisplatin resistance in gastric cancer by PI3K/AKT pathway (46). Similarly, Wang et al (2021) also reported SNHG9 upregulation is associated with DDP-resistance and poor prognosis of NSCLC patients(47). However, the role of SNHG9 in cisplatin chemoresistance was not studied. In this study, we performed in-vivo and in-vitro test to demonstrate the SNHG9 contribute for cisplatin resistance in hepatoblastoma cell lines (HepG2 & HUH6). A significant reduction in cell viability was reported in IC50 cisplatin treated SNHG9 siRNA knockdown HB cell in comparison to IC50 cisplatin SNHG9 overexpression plasmid transfected cell. A significant reduction in tumor size, volume and was reported in IC50 cisplatin SNHG9 siRNA injected mice. A significant increase in cellular apoptosis and cleaved-caspase-3 & 9 protein expression was reported in cisplatin treated HB cell in comparison to DMSO treated HB cell. These findings confirmed SNHG9 contributes cisplatin resistance in HB. However, the present study has certain limitations, due to the rarity of the diseases the number of the clinical samples taken for this study is low which may influence the research outcomes. We performed nuclear and cytoplasmic fractional separation method to elucidate the subcellular distribution of SNHG9 however, Fluorescence in-situ hybridization (FISH) was not done.

In Conclusion

In summary, to our acknowledgement our study is the first to explore the biological function and underlying molecular mechanisms of SNHG9 in hepatoblastoma tumorigenesis. Our study findings confirmed that SNHG9 acts as oncogene and promotes the HB tumorigenesis. Eventually, we elucidate that SNHG9 promotes the hepatoblastoma tumorigenesis via miR-23a-5p/Wnt3a axis. ROC analysis showed SNHG9 is high sensitivity and specificity identification of hepatoblastoma tumors thus it could be a potential diagnostic biomarkers and therapeutics targets for the treatment and early diagnosis of HB patients.

Abbreviations

lncRNAs: Long noncoding RNAs; HB: Hepatoblastoma; sAFP: Serum Alpha Fetoproteins; OS: Overall survival; qRT-PCR: Quantitative real-time polymerase chain reaction.

SDS-PAGE: Sodium Dodecylsulphate polyacrylamide gel electrophoresis; PMSF: phenylmethanesulfonyl fluoride; CCK8: Cell counting kit-8; HB: Hepatoblastoma. miRNAs: MicroRNAs. ceRNAs: competitive endogenous RNA, siRNA: short interfering RNA.

Declaration

Ethics and Committee Approval and Patient consents:

This study was approved by the Institutional Ethical committee of Shanghai Tenth People hospital affiliated to Tongji University of medicine (Approval no:2016-STPH-1203). All the Animals experiments were carried out following the approval by Animal protection and utilization committed of Tongji Hospital (No:TJ201202021167666STH). We declare that all the patients were participated in this study were aware about the purpose and content of this research. A verbal and written consents were taken prior to their participant in this study.

Consent for the publication:

We claim all authors have read and approved the final version submitted

Availity of data and materials:

The database used during the current study will be made available form the correspondence authors on requirements.

Competing Interest:

The authors declared that no competing interest exists.

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Author`s Contributions:

SF, MJ and PQ designed and supervised, RB performed the experiment and write manuscript writing, SG carried out the animal experiment, BZ, L Y, Z J, NZ, WJ, MS samples collection, editing's and data analysis.

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Tables

Tables 1: SNHG9 expression correlation with Clinicopathological characteristics of Hepatoblastoma patients.

Hepatoblastoma	SNHG9 Expression		χ ² value	p-value
	Low	High		
Age of diagnosis				
0-24 months	12	17	0.54	0.816
≥24 months	5	6		
Sex				
Male	6	5	1.338	0.247
Female	10	19		
AFB				
<1200ng/ml	5	5	0.889	0.346
≥1200ng/ml	10	20		
AFB Final detection				
<5 ng/ml	1	12	1.648	0.439
≥5ng/ml	5	7		
NA	1	5		
Histology				
Mixed	7	6	5.765	0.056
Epithelial		9		
NA	9	8		
PRETEXT				
I-II	2	7	2.079	0.354
III-IV	10	10		
NA	4	7		
Tumor Size				
<500 Cm3	3	10	3.301	0.192
≥500 cm3	13	13		
Metastasis				
YES	2	3	0.001	1
NO	14	21		
NA	0	0		

Figures

Figure 1

SNHG9 is significantly upregulated in hepatoblastoma tumors and Cell lines. (A-B) SNHG9 mRNA expression in hepatoblastoma tissue and cell lines (HUH6 & HepG2) was determined by the qRT-PCR. (C) Increased SNHG9 expression in SNHG9 Overexpression plasmid transfected HepG2 and HUH6 and was estimated by q-RT-PCR. (D) Decreased in SNHG9 expression on SNHG9 sirRNA knockdown HHU6 and Hepg2 cell. (E-F) Fractional distribution of SNHG9, GAPDH and U6 in cytoplasm and nuclear was determined by qRT-PCR and showed majority portion of SNHG9, GAPDH located in cytoplasm. *p-value<0.05, **p<0.01, ***p<0.0001.

Figure 2

Prognostic and diagnostic significance of SNHG9. (A). Kaplan-Meier analysis demonstrates, the patients with high SNHG9 expression had a poor overall survival rate (OS). (B) ROC curved analysis showed SNHG9 has a high degree of sensitivity and specificity in diagnosis of HB patients, making it a valuable diagnostic tool. ** $p<0.01$, *** $p<0.0001$.

Figure 3

(A-H): SNHG9 interferes in biological and apoptosis activity in hepatoblastoma. (A-B) SNHG9 overexpression plasmid transfected HUH6 & HepG2 showed increased in cellular proliferation and clonogenic activity. (C-D) SNHG9 siRNA knockdown HB cell lines showed a decreased in cell proliferation and clonogenic activity. (E) Increased in cellular apoptosis in SNHG9 knockdown HB cell lines. (F) Decreased cellular apoptosis activity in SNHG9 overexpressed hepatoblastoma cell lines was reported by Flow cytometry analysis. (G-H) Impact on the cellular apoptosis related protein expression on subsequent knock down and overexpression of SNHG9 on HB cell lines was estimated by Western blotting. *p-value<0.05, **p<0.01.

Figure 4

(A-F): SNHG9 promotes hepatoblastoma tumorigenesis via Wnt/β-catenin pathway. (A-B) Decreased relative expression of Wnt/β-catenin pathway related genes on SNHG9 knockdown HB cell lines. (C-D) Increased expression of Wnt/β-catenin signaling pathway related target genes in SNHG9 Overexpressed HB cell lines and was determined by qRT-PCR. (E) Decreased Wnt/β-catenin signaling pathway related target genes proteins seen in SNHG9 knock down HB cell lines. (F) Increased in Wnt/β-catenin pathway related target genes protein in SNHG9 overexpressed HB cell lines and was determined by Western blotting. *p-value<0.05, **p<0.01.

Figure 5

(A – H): miR-23a-5p deregulation promotes the hepatoblastoma tumorigenesis. (A-B) miR-23a-5p expression in hepatoblastoma tissue and cell lines determined by qRT-PCR. (C-D) The miR-23a-5p relative expression on miR-23a mimics and inhibitors transfected hepatoblastoma cell lines. (E-F) Decreased in cell proliferation and colony formation activity reported in miR-23a-5p transfected HB cell lines. (G-H) A enhanced cell proliferation and clonogenic activity was seen in miR-23a-5p transfected HB cell lines. *p-value<0.05, **p<0.01, ***p<0.0001.

Figure 6

(A-J): SNHG9 directly interact with miR23a-5p and negatively regulates miR23a-5p activity. (A) The bindings sites of miR23a-5p and SNHG9 was predicated by RNA-hybrid database. (B-G) Dual luciferase assay, RIP and biotin RNA Pull down assay to assess the association in between SNHG9 and miR-23a-5p (H-I) miR-23a-5p expression on SNHG9 overexpressed knockdown HB cell lines (J) Spearman`s correlation analysis to explore the correlation in between SNHG9 and miR-23a-5p. *p-value<0.05, **p<0.01, ***p<0.0001.

Figure 7

miR-23a-5p down regulates Wnt3a expression. (A) Wnt3a expression on hepatoblastoma tissue determined by qRT-PCR. (B) Relative expression of Wnt3a expression on HB cell lines transfected with miR-23a-mimics and inhibitors determined by qRT-PCR. (C) Wnt3a expression on SNHG9 overexpressed HB cell line. (D) Wnt3a protein expression in miR-23a mimics and inhibitors transfected cell. (E) Wnt3a protein expression in SNHG9OE and miR-23a-5p mimics co-transfected HB cell line (F) The miR-23a-5p interaction sites on Wnt3a by predicated RNA-hybrid database. (G-H) Linkage between miR-23a-5p and Wnt3a was confirmed by dual luciferase activities. (I) The Spemann's Correlation test showed the miR-23a-5p and Wnt3a showed the negative correlation. (J) Spemann's correlation showed the positive correlation in between SNHG9 and Wnt3a. *p-value<0.05, **p<0.01, ***p<0.0001.

Figure 8

SNHG9 knockdown inhibits hepatoblastoma tumorigenesis in vivo. (A) Decreased SNHG9 expression in Iv-shSNHG9 transfected Huh6 cell determined by qRT-PCR. (B) Knockdown of the SNHG9 efficiently inhibits the tumors growth in nude mice. (C-D) Tumor volume and weight. (E) Western blotting was used to detect Wnt/ β -catenin signaling pathway related protein (β -catenin, Wnt3a, c-Myc, survivin) from protein extracted from the tumor tissue isolated from six groups nude mice. Data are presented as the mean $\pm$ SD. ****p-value<0.0001.

Figure 9

SNHG9 promotes the cisplatin resistance in HB cell lines. (A-B) Cell viability of si-SNHG9 treated hepatoblastoma cell lines (HUH6 & HepG2) after subsequent treatment with IC50 cisplatin. (E-F) Cellular apoptosis of HB cells treated with IC50 cisplatin after transfection was determined by Flow cytometry analysis. (D) Western blotting analysis showed that high expression of cleaved caspase 3 and 9 activity in cisplatin treated HB cell in contrast to DMSO treated HB cells. (G) IC50Cisplatin and lvSHNG9 treatment significantly reduced the tumor growth. (H) Tumor growth on DMSO and Cisplatin treated mice. (I) Tumor volume of cisplatin and DMSO treated mice. *P=0.05, **P=0.001 and ***p=0.0001.

Supplementary Files

- [SupplementaryMaterials.docx](#)