MiR-125b-5p Inhibited The Progression of Hepatoblastoma As A Shared miRNA of The lncRNA NEAT1 and YES1

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Research

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Abstract

Background: microRNAs have been studied widely in hepatoblastoma. However, the role of miR-125b-5p and its relationship with the lncRNA sNEAT1 and YES1 in hepatoblastoma have not been reported previously. We aimed to reveal the role of NEAT1/miR-125b-5p/YES1 in the progression of hepatoblastoma.

Methods: We collected tumor tissues and their adjacent tissues from 12 hepatoblastoma patients. qRT-PCR was applied to detect the expression of miR-125b-5p, and the relationship of miR-125b-5p with clinicopathological characteristics was analyzed. Dual luciferase reporter assays and RNA pull down assays were used to identify the relationships among NEAT1, miR-125b-5p and YES1. CCK8, Transwell assays and wound healing assays were used to examine cell viability, invasion and migration. In vivo experiments were also applied to detect the effect of miR-125b-5p on hepatoblastoma.

Results: miR-125b-5p was significantly downregulated in hepatoblastoma tissue and cells. The higher the PRETEXT grade, the lower the miR-125b-5p level. NEAT1 could bind to miR-125b-5p and inhibit its expression. miR-125b-5p could target YES1 and inhibit its expression. Overexpression of miR-125b-5p decreased the proliferation, invasion, and migratory ability of hepatoblastoma cells. YES1 could rescue the above effects. At the same time, overexpression of miR-125b-5p resulted in decreased YES1 and tumor growth inhibition in vivo.

Conclusion: miR-125b-5p acted as a shared miRNA of NEAT1 and YES1 in hepatoblastoma. Overexpression of miR-125b-5p could target YES1 and inhibit its expression, therefore inhibiting the progression of hepatoblastoma.

Background

Hepatoblastoma (HB) is the most common malignant tumor of the liver in children. It often arises from liver progenitor cells or hepatoblasts during the embryonic development of the liver ¹. Hepatoblastoma accounts for approximately 1% of childhood cancers, and its incidence is approximately 1.5 per million ². With the improvement of infant survival rates and advances in treatment, the survival rate of children with the disease has increased significantly over the past few decades ^{3,4}. Due to the characteristics of hepatoblastoma, it is clinically unresectable in nearly half of all cases. With the introduction of new adjuvant chemotherapy, the size of the tumor can be reduced, allowing for more cases of resection ⁵. Despite improvements in diagnosis and treatment, the prognosis of the disease is still poor ⁶. At present, the role of many genes, lncRNAs and miRNAs in HB are being studied ^{7,8}, but the truly pathogenesis has not been revealed. Therefore, it is urgent for researchers to identify new candidate targets for the treatment of HB.

miRNAs are a type of small nonprotein-coding RNA comprised of approximately 20-25 nucleotides. They inhibit translation by binding to the 3'-UTR region of their target genes ⁹. Many miRNAs have been studied

in hepatoblastoma. Schmid et al.¹⁰ revealed that miR-492 significantly enhances the cell proliferation, migration, and invasion of hepatoblastoma cells. They also identified and validated that CD44 is a direct and functional target of miR-492. High miR-492 expression is correlated with high risk or aggressive tumors and has potential for predicting reduced event-free survival. A wide detection of miRNAs was applied by Olgun¹¹. They selected 4 miRNAs for that study, and they found only the level of miR-17 expression was significantly decreased in tumor samples. miR-125b usually acts as a tumor suppressor in many tumors, and it also protects damaged nerves¹²⁻¹⁴. Zhuo et al.¹⁵ reported that miR-125b suppresses the proliferation, migration, and invasion of Huh6 cells by targeting DKK3. Although the inhibitory effects of miR-125b on human cancer cell proliferation and metastasis have been extensively investigated¹⁶, the mechanism remains unknown.

YES proto-oncogene 1 (YES1) is a prominent member of the Src family of tyrosine kinases (SFKs). It acts as a regulator of tumor growth and is involved in the development of many human cancers¹⁷. In pancreatic cancer, inactivation of YES1 can significantly inhibit cell proliferation, invasion, and migration. YES1 can be regarded as an important therapeutic target in pancreatic cancer¹⁸. YES1 is a direct target of miR-128-3p and TUG1 modulates YES1 expression by sponging miR-128-3p. YES1 silencing repressed prostate cancer cell progression in vitro and mitigated tumor growth in vivo¹⁹.

There are no previous reports about YES1 in hepatoblastoma. To better understand the mechanism of hepatoblastoma progression, this study mainly explored the effects of miR-125b-5p and its possible target genes on hepatoblastoma. We aimed to explain the interaction between miR-125b-5p and YES1 in HB and provide more possible candidate targets for the treatment of HB.

Methods

Samples collected

We included 12 cases of hepatoblastoma who were operated on and confirmed by pathology in 2015 to 2016. All the patients were operated on before receiving radiotherapy, chemotherapy, or immunotherapy. The sample consisted of 7 boys and 5 girls, and their average age was 1.85 ± 1.46 . The adjacent tumor tissues from these patients were collected for controls. These hepatoblastomas were classified using the PRETEXT system²⁰. The expression level of miR-125b-5p was determined by ROC curves. Higher than the median was defined as high expression, lower was defined as low expression. All the patients were followed-up after treatment through December 2019. The survival time was calculated in months.

Cell proliferation assay

When the HepG2 and HuH-6 cells reached a logarithmic growth phase, we adjusted the cell concentration to approximately 1×10^4 /well in 96-well plates after digestion. The following day, the cells were transfected and then cultured for 24 h, 48 h, and 72 h. We added 10 μ l CCK-8 solution and incubated the

cells in the incubator for 2 h. The absorbance at 450 nm of each well was measured to draw the cell growth curve.

Cell invasion and wound healing assays

Cell invasion and wound healing assays were performed referenced to ⁸.

Dual luciferase reporter assay

Wildtype (WT) or mut sequences of the NEAT1 or YES1 3' UTR were synthesized and cloned into the pGL3-reporter vector. HepG2 or HuH-6 cells were co transfected with wild type or mutant plasmids and mir-125b-5p mimic or mimic NC vectors. After 48 h, we used a luciferase detection kit (#D0010, Solarbio, Beijing, China) and a dual fluorescent enzyme reporter gene analysis system (Promega, Madison, WI, USA) to detect luciferase activity.

RNA pull down assay

The RNA pull-down experiment was performed as described previously ²¹. The M-280 streptavidin beads were purchased from Thermo Fisher (#11205D, Thermo Fisher Science, Waltham, Massachusetts, USA). Purified RNA was used to detect the expression of NEAT1.

In vivo experiment

24 male BALB/c nude mice (purchased from Guangdong Medical Laboratory Animal Center) aged 5–6 weeks were randomly divided into a mimic NC group and a miR-125b-5p mimic group. Then, 0.2 ml 1×10^6 transfected cells were injected subcutaneously into each mouse's right armpit. We observed the mental diet and the body weight of the nude mice and recorded the tumor volume. When the tumor diameter reached 1.5 cm, the nude mice were killed, and the tumor was removed and weighed.

qRT-PCR

Total RNA was extracted from tissues and cells by TRIzol (#15596906, Thermo Fisher Science) and reversed transcribed into cDNA. The cDNA was used to apply qRT-PCR and U6 was set as the internal reference. Gene mRNAs were detected according to the TaqMan Gene Expression Assays protocol (#4331182, Thermo Fisher Science). GAPDH was used as the internal reference. The primer sequences are shown in Table 1. The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Lysis buffer (#P0013, Beyotime, Shanghai, China) was used to extract the total protein. Then, 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) were prepared, and the proteins were separated by electrophoresis and electro-transferred to polyvinylidene fluoride membranes, which were blocked with 5% skimmed milk for 1 h. We added the rabbit anti-YES1 primary antibody (#ab109744, 1:1000, Abcam, UK) and the anti- β -actin primary antibody (#ab8227, 1:5000, Abcam) separately and incubated them at 4

°C overnight. The secondary goat anti-rabbit IgG H&L antibody (#ab205718, 1:2000, Abcam) was added and incubated at 37 °C for 1 h. The results were shown by enhanced chemiluminescence (Thermo Fisher Science).

Statistical analysis

Data in our study were analyzed by SPSS 21.0 (SPSS, Inc, Chicago, IL, USA). Survival curve was drawn using Kaplan-Meier method and analyzed by Log-rank test. Data was shown in mean $\pm$ standard. Compared between two groups using t-test and multiple groups using ANOVA with Tukey's test for post hoc test. $P < 0.05$ indicated the difference was statistically significant.

Results

miR-125b-5p is expressed lower in hepatoblastoma and is related to prognosis

By qRT-PCR, we found that the expression of miR-125b-5p in cancer tissues was significantly lower than that in adjacent tissues ($P < 0.01$, **Figure 1A**). As shown in **Figure 1B**, we found that miR-125b-5p was expressed at lower levels in PRETEXT $\boxtimes$ – $\boxtimes$ grade ($P < 0.01$). However, the miR-125b-5p expression level had no relationship with age and gender. We analyzed the overall survival of the patients based on their expression of miR-125b-5p. The patients with high expression of miR-125b-5p had longer survival times than those with low expression of miR-125b-5p ($P = 0.013$, **Figure 1C**).

miR-125b-5p inhibited the progression of hepatoblastoma

To investigate the role of miR-125b-5p in hepatoblastoma, we compared the expression levels of miR-125b-5p in HepG2, HuH-6, and HL-7702 cells. **Figure 2A** shows that miR-125b-5p was expressed lower in HepG2 and HuH-6 cells compared with HL-7702 cells ($P < 0.05$). This result was consistent with the results from tissues. Then, we transfected miR-125b-5p mimic and mimic NC into the hepatoblastoma cells to observe the effect of miR-125b-5p. We found that the expression of miR-125b-5p was significantly higher in the miR-125b-5p mimic group ($P < 0.01$, **Figure 2B**). This indicated the transfection was successful. Through the cell proliferation assays, we found cells treated with the miR-125b-5p mimic had lower vitality ($P < 0.05$, **Figure 2C**). Their cell invasion and migration ability were also decreased (all $P < 0.05$, **Figure 2D, E**). These results suggested that miR-125b-5p significantly inhibited the progression of hepatoblastoma in vitro.

NEAT1 regulated the expression of YES1 by miR-125b-5p

Through the starBase website (<http://starbase.sysu.edu.cn/>), we found miR-125b-5p was a shared microRNA of NEAT1 and YES1. NEAT1 was overexpressed or knocked down in hepatoblastoma cells and then we detected the expression of miR-125b-5p by qRT-PCR. The results shown that compared to the Blank group, miR-125b-5p expression was increased in the si-NEAT1 group and decreased in the oe-NEAT1 group (all $P < 0.05$, **Figure 3A**).

Then, we explored the relationship between NEAT1 and miR-125b-5p in HepG2 cells using dual luciferase reporter gene assays. This suggested that the miR-125b-5p mimic significantly inhibited the relative luciferase activity of NEAT1-WT but had no effect on NEAT1-Mut ($P < 0.05$, **Figure 3B**). The results from RNA pull-down assays also indicated NEAT1 could combine with miR-125b-5p (**Figure 3C**). We cotransfected si-NEAT1 and a miR-125b-5p inhibitor to clarify the relationship between NEAT1 and YES1. qRT-PCR was applied to detect the expression of miR-125b-5p and YES1. When NEAT1 was knocked down, the expression of miR-125b-5p was upregulated but YES1 was downregulated. Silencing miR-125b-5p could reverse the interference of NEAT1 (**Figure 3D**). The above results implied NEAT1 inhibited the expression of miR-125b-5p by binding with miR-125b-5p, thus promoting the expression of YES1.

miR-125b-5p inhibited the growth of hepatoblastoma by targeting YES1

miR-125b-5p targeted to YES1 was predicted by TargetScan and we proved it through dual luciferase reporter gene assays (**Figure 4A**). When miR-125b-5p was overexpressed, the expression level of miR-125b-5p was increased more in the miR-125b-5p mimic group than in the mimic NC group. However, the expression level of YES1 was significantly decreased in the miR-125b-5p mimic group relative to the mimic NC group ($P < 0.05$, **Figure 4B**). miR-125b-5p mimic or Lv-YES1 were transfected into HepG2 and HuH-6 cells. Through qRT-PCR detection, we found that compared to the mimic NC+ Lv-NC group, miR-125b-5p was upregulated and YES1 was downregulated in the miR-125b-5p mimic+Lv-NC group ($P < 0.05$). The expression of YES1 was increased in the mimic NC+ Lv-YES1 group and this was reversed when cotransfected with miR-125b-5p mimic (**Figure 4C**). Next, we examined their cell proliferation, invasion, and migration ability separately (**Figure 4D-F**). According to these results, we found that the miR-125b-5p mimic could inhibit cell proliferation, invasion, and migration ability. However, YES1 enhanced these abilities and reversed the effect of miR-125b-5p.

In vivo, we found the tumor volume and tumor weight were significantly decreased in the miR-125b-5p mimic + Lv-NC group compared to the miR-125b-5p NC group. However, YES1 increased the tumor volume and tumor weight, and reversed the effect of miR-125b-5p ($P < 0.05$, **Figure 4G, H**). qRT-PCR was used to detect the expression of miR-125b-5p and YES1. This showed that compared with miR-125b-5p NC, the expression of miR-125b-5p was increased and that of YES1 was decreased in the miR-125b-5p mimic group ($P < 0.05$, **Figure 4I**). This suggested miR-125b-5p inhibited the expression of YES1 and played a role in inhibiting the tumor progression of hepatoblastoma in vivo.

Discussion

As a common malignant tumor among tumors of children, the prognosis of hepatoblastoma is often related to the type of disease differentiation. Although many genes and pathways have been studied in HB, the specific molecular mechanism has not been revealed²². It has been demonstrated that the miR-100/let-7a-2/miR-125b-1 cluster works with miR-371-3 in HB. Overexpression of the first cluster delays tumorigenesis and inhibits miR-371 while preventing tumorigenesis²³.

In this study, HB cancer tissues and cancer cells were used to examine the role of miR-125b-5p. It was found that compared with normal tissues and cells, miR-125b-5p was expressed at low levels in cancer tissues and cancer cells. Its low expression was related to the patient's PRETEXT grade and overall survival time. The lower its expression level, the higher the PRETEXT grade and the shorter the patient's overall survival time.

Previous studies of miR-125b-5p in tumor research have only reported limited results. miR-125b-5p has been studied the most in breast cancer. It can inhibit its target gene and its downstream pathways to inhibit the proliferation, invasion and migration of breast cancer cells, and improve their sensitivity to chemotherapy²⁴⁻²⁶. In ovarian cancer, the median serum levels of miR-125b in patients with epithelial ovarian cancer were significantly lower than that of controls. Serum miR-125b levels were found to be a useful diagnostic biomarker and a biomarker to predict the responses to chemotherapy in patients with epithelial ovarian cancer²⁷. In cervical cancer, it was found that LncRNA CAR10 upregulated PDPK1 to promote cancer development by sponging miR-125b-5p²⁸.

To better study the mechanism of miR-125b-5p in HB, we first explored it in HB cells. It was found that miR-125b-5p could significantly inhibit cell proliferation, invasion, and migration. Next, we used a bioinformatics website to predict that miR-125b-5p is a shared miRNA of the LncRNA NEAT1 and YES1 genes. LncRNAs regulate gene expression mainly at the epigenetic level, transcription level and posttranscription level²⁸. Transcription level and posttranscription level regulation are mainly mediated as molecular sponges, which inhibit the expression of target genes through binding and sponging miRNA²⁹. Through an RNA pull down experiment, we found that NEAT1 can bind to miR-125b-5p. YES1, as a target gene of miR-125b-5p, has reduced expression because of the interference of NEAT1. We speculate that NEAT1/miR-125b-5p/YES1 is a ceRNA mechanism that regulates HB development.

YES1, as a member of the SRC family, acts as an oncogene in most tumors³⁰ and plays an important role in the acquired drug resistance of breast and lung cancer^{31,32}. This study found that YES1 is significantly overexpressed in HB. Inactivation of YES1 can significantly inhibit the proliferation, invasion, and migration of HB cells. YES1, as the target gene of miR-125b-5p, can reverse the role of miR-125b-5p in HB. In vivo experiments also confirmed that miR-125b-5p can significantly inhibit the expression of YES1 and inhibit the growth of tumors. Yes-associated protein (YAP)1 has been reported to be related to tumor development and it cooperates with other signaling pathways. It has been reported that Wnt/ β -catenin works in association with Hippo/YAP to induce the development of hepatoblastoma^{33,34}. We suspect that miR-125b-5p targets and regulates YES1, causing changes in related signaling pathways that are involved in the progression of HB.

Overall, this study confirmed that miR-125b-5p is significantly under expressed in HB. The expression level of miR-125b-5p is related to the PRETEXT stage and the patient's overall survival time. miR-125b-5p is a shared miRNA of NEAT1 and YES1. NEAT1 binding to miR-125b-5p targets the regulation of YES1. The NEAT1/miR-125b-5p/YES1 axis is involved in HB disease progression. miR-125b-5p and YES1 may provide new targets for the treatment of HB.

List Of Abbreviations

HB, Hepatoblastoma; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gels; SFKs, Src family of tyrosine kinases; WT, Wildtype; YAP, Yes-associated protein; YES1, YES proto-oncogene 1.

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of the Affiliated Hospital of Zunyi Medical University and conducted ethically in accordance with the Helsinki Declaration. All animal experiments and care were performed according to the Animal Care and Treatment Administration of the National Ministry of Health and the requirement of the Ethics Committee in The Affiliated Hospital of Zunyi Medical University.

Consent for publication

The authors affirm that human research participants provided informed consent for publication.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yuanmei Liu contributed to the study conception and design. Substantial contributions to the conception or design of the work was made by Zhu Jin and Yutong Chen. Material preparation, data collection and analysis were performed by Mingjuan Gao, Zebing Zheng, Chengyan Tang, Lu Huang, and Yan Qu. The first draft of the manuscript was written by Yuchen Mao and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. Primer sequences for qRT-PCR

Gene	Sequences
miR-125b-5p	Forward: 5'TCCCTGAGACCCTAACTTGTA-3'
	Reverse: 5'- CTAATACGACTCACTATAGGGC-3'
U6	Forward: 5'-GCTTCGGCAGCACATATACTAAAT-3'
	Reverse: 5'- CGCTTCACGAATTTGCGTGTCAT-3'
NEAT1	Forward: 5'-ATGCCACAACGCAGATTGAT-3'
	Reverse: 5'- CGAGAAACGCACAAGAAGG-3'
YES1	Forward: 5'-CTAGCTAGCATGGGCTGCATTAAAAGTAAAG-3'
	Reverse: 5'- CTAGTCTAGATTATAAATTTTCTCCTGGCTG-3'
GAPDH	Forward: 5'-AAATGGTGAAGGTCGGTGT-3'
	Reverse: 5'- CCTGGAAGATGGTGATGGG-3'

Figures

Figure 1

Low expression of miR-125b-5p in hepatoblastoma. A, the expression levels of miR-125b-5p in hepatoblastoma tissues and adjacent tissues were detected by qRT-PCR (N=12). B, the expression of miR-125b-5p at different ages, sexes, and PRETEXT grades. C, the expression of miR-125b-5p and the prognosis of hepatoblastoma analyzed by Kaplan–Meier curves. The experiment was repeated three times. Data are expressed as the mean $\pm$ standard deviation, and a t-test was performed.

Figure 2

miR-125b-5p inhibited hepatoblastoma cellular proliferation and invasion ability A, expression of miR-125b-5p in HepG2, HuH-6 and HL-7702 detected by qRT-PCR. B, expression of miR-125b-5p in the different transfected groups. C, the role of miR-125b-5p in cell proliferation of the hepatoblastoma cells. D, the cell invasion ability as determined by Transwell assays ($\times 200$). E, Cell migration distance detected

by a scratch test. Every experiment repeated three times and expressed as mean $\pm$ standard deviation; comparisons between two groups were analyzed using independent t test, and comparisons among multiple groups were analyzed using one-way ANOVA. * indicates $P < 0.05$. ** indicates $P < 0.01$.

Figure 3

NEAT1 regulated the expression of YES1 through miR-125b-5p A, expression of miR-125b-5p when cells were treated with overexpressed NEAT1 or knocked down NEAT1. B, relationship between NEAT1 and miR-125b-5p detected by dual luciferase reporter gene assays. C, RNA pull down was applied to identify the relationship between NEAT1 and miR-125b-5p. D, expression of miR-125b-5p and YES1. Every experiment repeated three times and expressed as mean $\pm$ standard deviation; comparisons among multiple groups were analyzed using one-way ANOVA. * indicates $P < 0.05$. ** indicates $P < 0.01$.

Figure 4

miR-125b-5p inhibited tumor growth of hepatoblastoma by targeting YES1 in vivo A, TargetScan predicted and dual luciferase verified the relationship of miR-125b-5p and YES1. B, expression of miR-125b-5p and YES1 when miR-125b-5p was overexpressed. C, expression of YES1 in different transfected groups. D, cell proliferation detected by CCK8. E, the cell invasion ability determined by Transwell assays ($\times 200$). F, cell migration distance detected by a scratch test. G, tumor volumes in different groups. H, tumor weight in different groups. I, expression of YES1 in vivo. Every experiment repeated three times and expressed as mean $\pm$ standard deviation; comparisons between two groups were analyzed using independent t test, and comparisons among multiple groups were analyzed using one-way ANOVA. * indicates $P < 0.05$ compared with mimic NC+Lv-NC. # indicates $P < 0.05$ compared with miR-125b-5p mimic+Lv-NC. & indicates $P < 0.05$ compared with mimic NC+Lv-YES1.