EXPLORING ROLES OF MIR-885-5P IN HEPATOCELLULAR CARCINOMA

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ABSTRACT

Hepatocellular carcinoma (HCC), the most prevalent form of liver cancer, affects a variety of populations with a high impact in Southeast Asian countries, ranking as the third leading cause of cancer-related mortality worldwide. Lack of current effective therapeutics results in a low survival rate and a high recurrence rate. The development of novel, effective therapies required better knowledge of the disease's pathogenesis. MicroRNAs (miRNAs) were found to play an important role in tumorigenesis, including in HCC. Thus, we compared the expression levels of miRNAs in tumors with normal tissue and in tumor stage III with stage I. Among many miRNAs, hsa-miR-885-5p was selected for investigation. We show that hsa-miR-885-5p is a tumor suppressor in HCC by inhibiting cell proliferation, possibly by suppressing CDK6 expression. Hsa-miR-885-5p may exert its tumor suppressor role by targeting genes involved in cell cycle processes.

Keywords: miRNAs, Hepatocellular carcinoma, tumor suppressor

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most frequently diagnosed type of liver cancer, accounting for around 90 percent of all cases (Llovet et al., 2021). Nearly all cirrhosis-inducing conditions can cause HCC. These include prolonged hepatitis B and C virus (HBV and HCV) infection, chronic alcohol consumption, an aflatoxin-B1-contaminated diet, and non-alcoholic fatty liver disease (Farazi & DePinho, 2006). World Health Organization (WHO) estimated that more than 905,000 individuals had been diagnosed with the disease and more than 830,000 had died in 2020. Liver cancer was placed sixth in terms of incidence and third in terms of death when compared to other types of cancer (Sung et al., 2021). Although therapy for HCC has made significant progress, many patients still have a low 10-year survival rate and a high likelihood of 5-year recurrent HCC (Xu et al., 2021). Therefore, understanding the pathogenesis of HCC is crucial to the development of novel treatments.

MicroRNAs (miRNAs) are a class of small, noncoding RNAs that regulate gene expression. The formation of canonical miRNAs involves several processes (Shrivastava et al., 2015). MiRNAs are transcribed into primary miRNAs (pri-miRNAs) by RNA polymerase II, then processed by Drosha and Dicer to produce a mature miRNA duplex. The mature miRNA is loaded into Argonaute (AGO) proteins, which mediate target mRNA recognition and suppression of target gene expression (Shrivastava et al., 2015). Many studies have discovered the association between alterations in the expression of miRNAs and many cancers, including HCC (Uzuner et al., 2022). As a result, miRNA dysregulation might have a significant impact on the pathophysiological processes of human malignancies.

My bioinformatic analysis revealed that hsa-miR-885-5p was downregulated in the tumor tissue and normal tissue of HCC patients. As mentioned before, cirrhosis-inducing conditions can cause HCC (Farazi & DePinho, 2006). Hepatic stellate cells (HSCs) activation is the major biological process that plays an important role in hepatic fibrosis development (Zhang et al., 2016), and preliminary results show the potential anti-fibrotic role of hsa-miR-885-5p. In this study, I will further investigate the role of hsa-miR-885-5p in HCC.

Current knowledge on HCC is limited, particularly regarding miRNAs and their roles in HCC. This research attempts to advance our understanding of miRNAs and their function in HCC by exploring the association of hsa-miR-885-5p with HCC.

LITERATURE REVIEWS

Primary liver cancer

Primary liver cancer is the sixth most prevalent cancer in the world, accounting for 4.7% of all cancer-related incidents and 8.3% of all cancer-related mortality (Sung et al., 2021). In developed nations, the disease is not very prevalent, with an estimated 22.1% of all new cases. Contrarily, liver cancer is highly prevalent in the developing world (77.9% of new cases), with a particularly high incidence and mortality rate in Sub-Saharan Africa, East and Southeast Asia, Melanesia, and Micronesia/Polynesia (Ferlay J, 2020). This is mostly because chronic hepatitis B virus (HBV) infection and eating foods contaminated with the fungal toxin aflatoxin B1 are the two most important environmental risk factors for liver cancer (Zhang et al., 2017). Both occur much more often in low-income countries (Liu & Wu, 2010). In Thailand, liver cancer is the most common type of cancer. In 2020, there are expected to be 27,394 new cases. It is the most common cancer among men and the fourth most common among women. Furthermore, liver cancer is the leading cause of cancer death in both males and females in Thailand, with 26,704 people dying in 2020 (Ferlay J, 2020).

Hepatocellular carcinoma (HCC)

The majority of primary liver cancers are dominated by HCC. Geographical differences affect the distribution of HCC due to the variability of underlying risk factors (Samant et al., 2020). HCC was expected to occur in 72% of the countries in Asia. Globally, Thailand is one of the

four Southeast Asian countries that rank among the top ten countries with the highest incidence rates (Ferlay J, 2020). Incidence and mortality rates are approximately identical as a consequence of the poor prognosis, the associated underlying liver disease, the aggressiveness of the cancer, and the limited therapeutic options (Suresh et al., 2020). HCC exhibits a clear predilection toward men. In low-incidence areas, men are four times more likely than women to develop HCC. In high-incidence areas, HCC is eight times more likely to occur in men than in women (Leong & Leong, 2005). Chronic liver disease is observed in more than 90% of HCC cases (Llovet et al., 2021). The greatest risk factor for HCC is cirrhosis of any etiology. HCC is the predominant source of mortality in cirrhotic patients.

Current treatment for HCC

The choice of treatment for HCC will depend on several factors, including the size and location of the cancer, the stage of the cancer, and the patient's overall health (Raza & Sood, 2014). Some of the treatment options for early and intermediate HCC include surgery, liver transplantation, ablation, and embolization. For advanced HCC, chemotherapy, targeted therapy, and immunotherapy are used to treat patients (Shannon et al., 2022).

Although current treatment for HCC has improved in recent years, it is still often challenging to cure the disease. While these treatments can be effective in controlling the symptoms of HCC and extending life expectancy, they are generally not able to cure the disease. As a result, it is important for people with HCC to receive ongoing care and monitoring to manage the disease and its complications.

miRNAs and HCC

MiRNAs are small, non-coding RNA molecules of ~23 nucleotides that play important roles in the regulation of gene expression by binding to specific target messenger RNAs (mRNAs) and inhibiting their translation into proteins (Bartel, 2009). By forming a miRISC with the AGO protein family, miRNA can bind to the 3' untranslated region (UTR) of target mRNA and cause mRNA degradation and/or translational repression (Hammond, 2015). Dysregulation of miRNA expression has been implicated in the development and progression of various diseases, including HCC, by altering gene expression involved in carcinogenesis.

Certain miRNAs have been found to be consistently upregulated or downregulated in HCC, indicating that they may play a role in the disease's development and progression. MiRNAs have been demonstrated to affect several HCC processes, including cell proliferation, apoptosis, angiogenesis, epithelial-mesenchymal transition, drug resistance, autophagy, and metastasis (Xu et al., 2018). As a result, miRNAs are being investigated as possible therapeutic targets for HCC therapy. A better understanding of the role of miRNAs in the disease will lead to the development of new treatments for HCC. However, more research is needed to fully understand the role of miRNAs in HCC and determine their potential as targets.

RESEARCH METHODOLOGY

MicroRNA expression profile analysis

The expression profiles of miRNAs in HCC were retrieved using the TCGA and GEO databases. In the GEO database, GSE147889 was chosen to perform differential expression analysis. Next, analyze the expression profile from the TCGA database between normal tissue and a primary tumor and between HCC stages I and III using TCGAbiolinks. For the survival analysis, expression levels and survival times of patients from the TCGA database were retrieved to perform a Kaplan-Meier survival analysis.

Cells

Cell lines SNU-449, HepG2, THLE-2, and HEK293T were purchased from the American Type Culture Collection (ATCC). JHH-4 was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank. The Stbl3™ E. coli strain was provided by Thermo Fisher Scientific. HepG2 cell line was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM)

with low glucose containing 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% antibiotic-antimycotic (Gibco™, Thermo Fisher Scientific), while HEK293T was maintained in DMEM with high glucose medium supplemented with 10% FBS, 1% L-glutamine, and 1% antibiotic-antimycotic. JHH-4 was maintained in Eagle's Minimum Essential Medium (EMEM) (Gibco™, Thermo Fisher Scientific) supplemented with 10% FBS, 1% L-glutamine, and 1% antibiotic-antimycotic. THLE-2 was cultured in Bronchial Epithelial Cell Growth Medium (BEGM) with 5 ng/mL EGF, 70 ng/mL phosphoethanolamine (Lonza, Walkersville, MD), and 10% FBS. All cells were incubated at 37 °C with 5% CO_2.

MiRNAs from three cancer cell lines and one normal hepatocyte cell line were extracted using the GenUP™ Micro RNA Kit (Biotechrabbit) according to the manufacturer's protocol. The miRNAs were then reverse transcribed to cDNA by using 10xNEBuffer (New England Biolabs) 2.5 µl, 50 mM UTP 0.25 µl, 40U/µl RiboLock (Thermo Fisher Scientific) 1 µl, 500 ng miRNA, and nuclease-free water to a final volume of 25 µl. The mixture was incubated for 10 minutes at 37 °C. 0.4 µl of 10 µM SL-PolyA was then added to the mixture and incubated at 65 °C for 5 minutes. Then, the reaction was cooled on ice for 2 minutes. Next, 5xRT Buffer (Thermo Fisher Scientific) 8 µl, 10 mM dNTPs 4 µl, 200U RevertAid 2 µl, and 40U/µl RiboLock 1 µl was added to the mixture and incubated at 42 °C for 60 minutes, followed by heat inactivation at 70 °C for 10 minutes. The final qPCR reaction mixture, of a 10 µl final volume, contained 2.5 µl of 4X CAPITAL™ qPCR Probe Master Mix (Biotechrabbit), 0.25 µl of each 10 µM primer, and 6 µl of nuclease-free water. The conditions of qPCR were 95 °C for 10 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 55 °C for 15 seconds, and extension at 72 °C for 20 seconds, all performed on a Life Technologies QuantStudio™ 3 real-time PCR system. The primers used in this study are shown in Table 1.

Table 1 Primer's sequence used for miRNA expression analysis

miRNA name	Forward primer (5' → 3')	Reverse primer (5' → 3')
U6	CTCGCTTCGGCAGCACA	GCAGGGTCCGAGGTATTCG
hsa-miR-885-5p	TCCATTACACTACCCTGCCTCT	GCAGGGTCCGAGGTATTCG

Overexpression of miRNA

To study the gain-of-function of hsa-miR-885, the sequence of the pri-miRNA of hsa-miR-885 and its 100-200 bps upstream and downstream sequences were amplified from human genomic RNA using PCR. The purified PCR product was ligated into the pLV-EF1α-IRES-Puro (Addgene Plasmid #85132) that was digested with EcoRI and BamHI. Next, the vector was used to transfect the HCC cell line using Lipofectamine 3000 transfection reagent (Invitrogen), according to the manufacturer's protocol. After 48 hours, the media was removed and replaced with 2 ml of complete media containing 1, 2, and 5 µg/ml of puromycin for selecting HepG2, JHH-4, and SNU-449 cells transfected with the hsa-miR-885 plasmid, respectively. The expression levels of hsa-miR-885-5p were analyzed using qPCR as previously described.

MTT assay for cell proliferation

HCC cell lines ($5x10^4$ cells/ml) were seeded onto 96-well plates containing a final volume of 100 µl/well and incubated for the desired period. MTT formazan (Sigma-Aldrich) was dissolved in DMEM with low glucose to provide a 1 mg/100 µl stock solution. The stock solution was diluted with low-glucose DMEM to make an MTT solution of 0.5 mg/ml. In each well, the culture medium was discarded and replaced with 100 µl of 0.5 mg/ml MTT solution, incubated for 30 min at 37 °C with 5% CO_2, and then discarded. Next, 100 µl of DMSO (Sigma-Aldrich) was added, incubated for 5 min at 37 °C with 5% CO_2, and thoroughly mixed to ensure complete solubilization. Finally, all the samples' absorbances were measured at 570 nm using a BioTek™ Synergy™ HTX Multi-Mode Microplate Reader (Thermo Fisher Scientific).

BrdU assay

First, a 10 mM BrdU stock solution was diluted in cell culture medium to produce a 10 µM BrdU labeling solution. Prior to the experiment, cells were seeded into a 24-well plate at a concentration of 1 x 10^5 cells/ml and incubated for 48 hours without disturbance. The cell medium was replaced with 500 µl of a BrdU labeling solution containing 10 M, and the cells were incubated as needed at 37 °C and 5% CO_2. After removing the labeling solution, the cells were washed three times for two minutes with PBS. After removing the PBS from the culture, 500 µl of the 4% formaldehyde in the PBS were added. 15 minutes were spent incubating cells at 37 °C and 5% CO_2 before they were washed three times for two minutes with PBS. Once PBS was removed, 500 µl of Triton X-100 permeabilization buffer were added. The cells were incubated at room temperature for 20 minutes prior to the removal of the permeabilization buffer and the addition of 500 µl of 2N HCl, respectively. The cells were cultured for 30 minutes at room temperature. After the removal of 2N HCl, three 2-minute washes with permeabilization buffer were performed. Following the addition of 500 µl of blocking buffer (5% BSA in the permeabilization buffer), the cells were incubated at room temperature for 30 minutes. To the culture was added 500 µl of blocking buffer containing anti-BrdU primary antibody (sc-32323; Santa Cruz) at a 1:200 dilution. After an overnight incubation at room temperature, cells were washed three times for five minutes with permeabilization buffer. The cells were incubated at room temperature for one hour after the addition of secondary antibodies (1:500 dilution) and DAPI (1:1000 dilution). The culture was washed with permeabilization buffer three times for five minutes before the addition of PBS. A fluorescent microscope was used to examine the cells.

Sample preparation for RNA-Seq

Mir-885-transfected JHH-4 cells were seeded into 2 wells of a 24-well plate and cultured until they reached confluence. Then, the cells were harvested for RNA extraction using the RNeasy Mini Kit (Qiagen) with on-column DNA digestion, following the manufacturer's protocol. At least 3 µg of total RNA was loaded into GenTegra-RNA 0.5 mL screw-cap microtubes (GenTegra) and vacuumed until dry. The Agilent Bioanalyzer 2100 system (Agilent Technologies) was used to test RNA integrity. The NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs) was used to prepare the library using 1 µg of total RNA with a RIN value greater than 7. The library was sequenced with the NovaSeq 6000 System (Illumina). The NGS service and data analysis were performed by Biomarker Technologies (BMKGene). Further data analysis was performed to identify the potential candidate target.

Dual luciferase assay

The first step is to clone the 3' UTR sequences into the pmiRGLO vector using SacI and XhoI sites and the hsa-miR-885-5p sequence into the pSilencer 3.0-H1 vector using BamHI and HindIII sites. Then we transfected the 1x10^4 HEK293FT cell lines with renilla-firefly plasmid and hsa-miR-885-5p using jetPRIME® transfection reagent according to the manufacturer's protocol. There are three groups of plasmids. The first contains the 3' UTR of the interested mRNA target downstream of firefly luciferase. The second plasmid contained mutant 3' UTRs of interested mRNA targets, and the final plasmid is a control plasmid that does not have 3' UTR inserts of interested mRNA targets. Next, using the Dual-Luciferase® Reporter Assay kit (Promega), the cells were lysed using 20 µl of Passive Lysis Buffer (PLB), and the plate was shaken for 15 minutes at room temperature. Then, add 50 µl of LAR II to each well and measure luminescence to observe firefly luciferase activity with the BioTek™ Synergy™ HTX Multi-Mode Microplate Reader (Thermo Fisher Scientific), add Stop&Glo® reagent to terminate firefly luciferase activity, and measure renilla luciferase activity.

Statistical analysis

Data analyses were performed using with GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA) to performed t-test, p value <0.05 was considered statistically significant.

RESEARCH RESULTS

Hsa-miR-885-5p expression profile in cancer patients

In the GEO database, expression profiles of miRNAs from GSE147889 indicate that 105 miRNAs were found to be significantly downregulated when logFC was less than -0.5 and the adjusting p-value was less than 0.05. The results from the TCGA database indicated that 234 miRNAs were downregulated when comparing HCC tissue to normal tissue, and 67 miRNAs were downregulated when comparing stage III HCC to stage I HCC. After matching the miRNAs from both databases, nine miRNAs were found to overlap (Figure 1a). Next, narrow down the list of miRNAs by using TargetScan to predict miRNA targets. Hsa-miR-885-5p, which has the highest number of potential targets (3082 sites), was selected. By using survival data in the TCGA database, high expression of hsa-miR-885-5p was found to be associated with a high survival rate (Figure 1e), which further confirms the tumor suppressor role of hsa-miR-885.

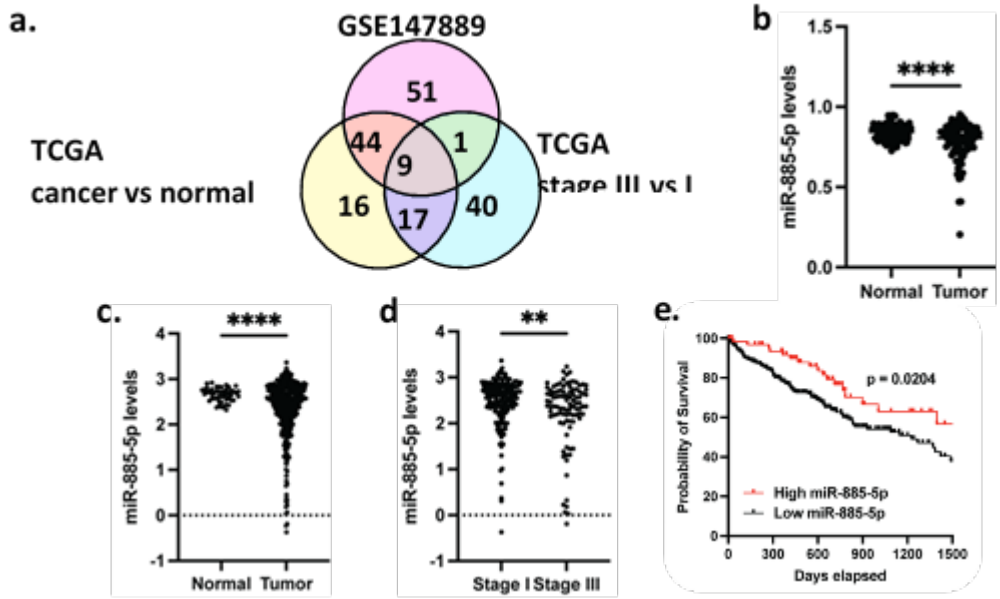

Figure 1 Preliminary analysis of downregulate miRNAs in HCC. (a) Venn diagram of downregulate miRNAs from GEO and TCGA database. (b) Expression level of hsa-miR-885-5p between normal and tumor tissue in GSE147889. (c) Expression level of hsa-miR-885-5p between normal and tumor tissue in TCGA database. (d) Expression level of hsa-miR-885-5p between HCC stages III and I in the TCGA database. (e) Kaplan-Meier curve of hsa-miR-885-5p in HCC.

Hsa-miR-885-5p expression profile in HCC cell lines

To determine the role of hsa-miR-885-5p in the carcinogenesis of HCC, we analyzed the expression profiles of hsa-miR-885-5p in HCC cell lines compared with normal hepatocyte cell line using RT-qPCR. Figure 2 shows that the expression of hsa-miR-885-5p was lower in HCC cell lines (HepG2, JHH-4, and SNU-449) than in THLE-2, which is used as a normal hepatocyte cell line. The expression profiles indicate the highest downregulation of hsa-miR-885-5p in JHH-4 cell lines, with an 82.73-fold lower level than THLE-2, followed by HepG2 and SNU-449 cell lines (6.47 and 1.51-fold, respectively). This result suggests that hsa-miR-885-5p may act as a tumor suppressor.

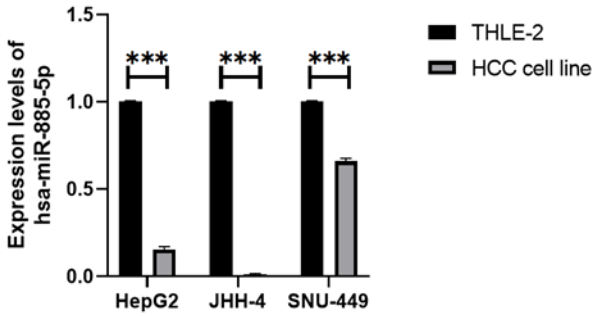

Figure 2 Expression level of hsa-miR-885-5p in HCC cell lines (HepG2, JHH-4, and SNU-449) compares with that in normal hepatocyte cell line (THLE-2). ***p <0.0001

Hsa-miR-885-5p suppresses the growth of HCC cells *in vitro* To further confirm the tumor suppressor function of hsa-miR-885-5p, we established hsa-miR-885-expressing HCC cell lines. Following the selection with puromycin until the negative control disappeared, we confirmed the overexpression of hsa-miR-885-5p by RT-qPCR. The expression levels of hsa-miR-885-5p were indeed upregulated in hsa-miR-885-overexpressed cell lines, with 6.17, 58.2, and 49.64-fold increases in HepG2, JHH-4, and SNU-449, respectively (Figure 3a), which indicated successful delivery and expression of hsa-miR-885. HCC-bearing hsa-miR-885 cells showed a significant decrease in cell proliferation compared with control as determined by MTT assay (Figure 3b-d), further supporting the tumor suppressor role of hsa-miR-885-5p.

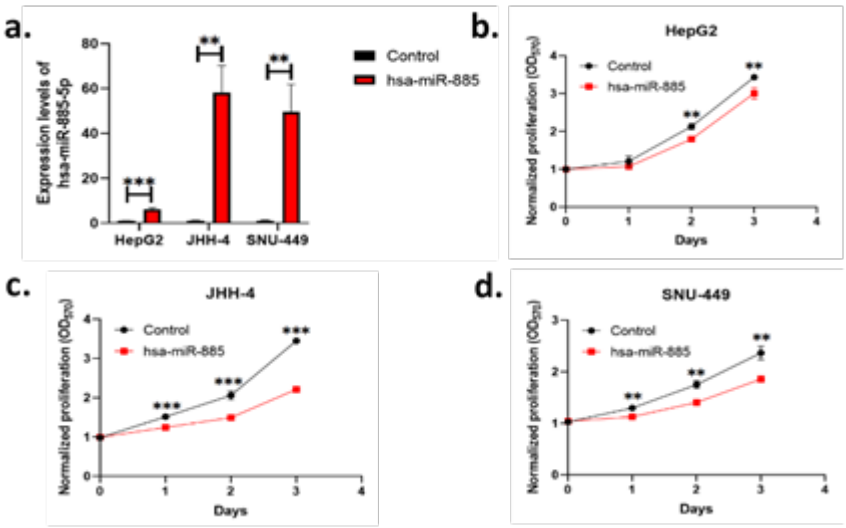

Figure 3 Hsa-miR-885-3p attenuates cells growth *in vitro*. (a) qPCR-based quantification of relative expression of hsa-miR-885-5p in hsa-miR-885-overexpressed HCC cell lines compared with control. (b-d) Proliferation rate of HCC cell lines (HepG2, JHH-4, and SNU-449, respectively). **p <0.001 and ***p <0.0001

Hsa-miR-885-5p inhibits the G1/S transition of HCC cells

As MTT assays indicate a proliferative inhibitory effect of hsa-miR-885-5p, we suspect that hsa-miR-885-5p can prevent cell cycle progression, especially G1/S transition, which is crucial for the control of eukaryotic cell proliferation. To confirm this hypothesis, we analyzed cells in S phase using the BrdU incorporation assay. As shown in Figure 4, the results suggested that hsa-miR-885-5p could decrease G1 phase progression in SNU-449, additionally verifying the antiproliferative effect of hsa-miR-885-5p.

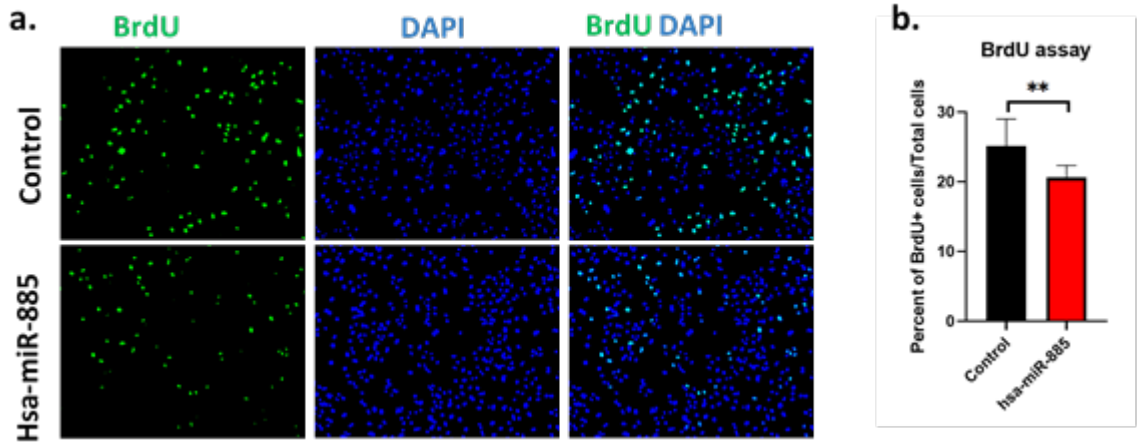

Figure 4 BrdU incorporation assay. (a) Representative fields of cells are shown stained with anti-BrDU (top panels) or DAPI (middle panels), with merged images shown in the bottom panels. (b) Quantitative data showing the percentage of BrDU-positive cells. **p <0.001

RNA-seq analysis of JHH-4 overexpressing hsa-miR-885

We used RNA-seq to unbiasedly identify the potential mRNA targets of hsa-miR-885-5p. There are a total of 797 annotated genes among the 990 downregulated genes. To further explore the function of the identified target genes, GO term, KEGG, and Reactome pathway analyses were performed (Figure 5). The results showed that many genes were mainly enriched in cell cycle processes, thus further confirming the tumor suppressor role of hsa-miR-885-5p.

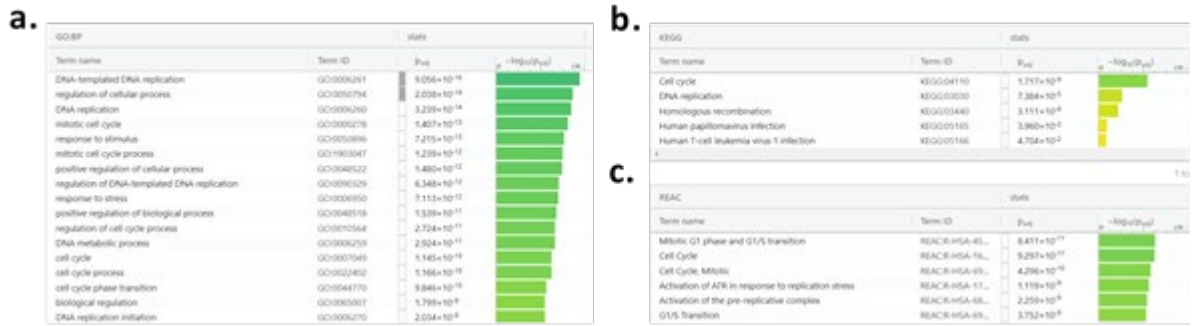

Figure 5 GO term and pathway analysis of genes obtained from RNA-seq comparison between JHH-4 expressing hsa-miR-885 and control**.** (a) Biological Processes (BP) category of the GO term. (b) KEGG enrichment analysis. (c) Reactome pathway analysis.

Cyclin Dependent Kinase 6 (CDK6) is a direct target of hsa-miR-885-5p

From Figure 5c, the G1/S transition was the most significant pathway that was enriched. This was also confirmed by the BrdU incorporation assay. To identify the potential target of hsa-miR-885-5p, the top 10 genes that were downregulated in RNA-seq analysis and involved in the G1/S transition were used to predict the possible interaction with hsa-miR-885-5p using TargetScan (Figure 6a). Among the 10 targets, E2F2 and CDK6 may interact with hsa-miR-885-5p, with CDK6 showing the most potential as a target. We further analyzed the interaction between hsa-miR-885-5p and CDK6 by a dual-luciferase assay. The sequences of hsa-miR-885-5p, the wild-type 3' UTR of CDK6, and the mutant 3' UTR of CDK6 are illustrated in Figure 6b. The results indicated that hsa-miR-885-5p could directly target CDK6 and suppress its expression, and when the 3' UTR of the CDK6 sequence was mutated, hsa-miR-885-5p was no longer able to inhibit the expression of CDK6 (Figure 6c).

a.

mRNA	FC (miR-885 vs control)	p-value
E2F2	-1.674874926	2.34E-58
MYBL2	-1.403213276	2.66E-116
MCM10	-1.087550506	1.51E-63
POLE2	-1.014576446	1.36E-13
PPP2R1B	-1.008936561	1.13E-59
TK1	-0.998236331	3.03E-58
CDK6	-0.968297487	6.02E-120
RRM2	-0.967096287	1.69E-121
MCM6	-0.952808085	2.64E-67
TYMS	-0.952383501	4.12E-83
CDC7	-0.941838797	2.72E-27
CDC6	-0.934696391	6.04E-88
CDC45	-0.934505343	1.19E-55
MCM3	-0.931881933	4.13E-72

b.

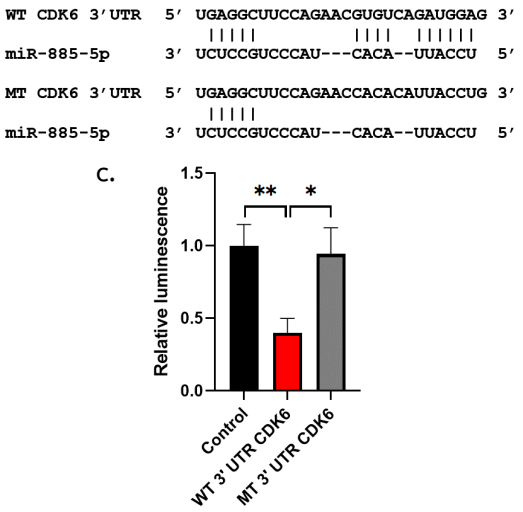

Figure 6 *CDK6* is a direct target of hsa-miR-885-5p. (a) Log_2fold change of genes that were downregulated in RNA-seq analysis and involved in the G1/S transition process. Possible targets are shown in red. (b) Sequences of hsa-miR-885-5p, the wild-type 3' UTR of CDK6, and the mutant 3' UTR of CDK6. (c) Dual-luciferase reporter assay. *p <0.05 and **p <0.01

DISCUSSION & CONCLUSION

To identify candidate tumor suppressor miRNAs, we used bioinformatic analysis. The expression profile of hsa-miR-885-5p was reduced in HCC tissue when compared to normal tissue in both the GEO and TCGA databases (Figure 1b, c). Moreover, higher-grade tumors were found to have lower expression of hsa-miR-885-5p (Figure 1d), indicating their potential antitumor role, which is additionally supported by the Kaplan-Meier survival analysis (Figure 1e). Among the 9 miRNAs that were downregulated in our preliminary analysis, hsa-miR-885-5p has the highest predicted target, giving a vast majority of targets for investigation and a higher chance for novel target discovery.

We verified the downregulation of hsa-miR-885-5p in three HCC cell lines (HepG2, JHH-4, and SNU-449) (Figure 2). Thus, we were able to use these cell lines to further investigate the role of miRNA in HCC. Indeed, hsa-miR-885-5p exerts a tumor suppressor role by suppressing cell proliferation in both MTT (Figure 3) and BrdU (Figure 4) assays. BrdU incorporation assays also demonstrate the role of hsa-miR-885-5p in preventing the G1/S transition, which initiates cell proliferation.

Previous studies have also found that hsa-miR-885-5p can suppress tumors in many cancers, including neuroblastoma (Afanasyeva et al., 2011), gastric cancer (Jiang et al., 2021), and HCC (Li et al., 2020). To gain insight into the molecular mechanism of hsa-miR-885-5p, we performed RNA-seq analysis of JHH-4 expressing hsa-miR-885-5p. The results indicate the important role of hsa-miR-885-5p in cell cycle processes (Figure 5). Cell cycle processes are important for cancer. The aberration in cell cycle regulatory proteins has been found in virtually all human cancers (Liu et al., 2022). Thus, miRNAs that target proteins in this process may exert potential therapeutic effects for HCC. We confirmed that CDK6, a gene involved in cell cycle processes, was the direct target of hsa-miR-885-5p.

Due to insufficient time to conduct other experiments, we were unable to further study CDK6 and gain access to its role in HCC. We plan for future studies to focus on CDK6 by analyzing its protein expression with western blotting. Moreover, our BrdU incorporation assay only has results from SNU-449, so we plan to perform experiments in HepG2 and JHH-4.

In summary, our study revealed that hsa-miR-885-5p overexpression can inhibit cell proliferation. RNA-seq analysis revealed the significant role of hsa-miR-885-5p in cell cycle processes, which are essential for the development of cancer and are the focus of many cancer therapies. Our luciferase assay also verified CDK6, which plays a role in the cell cycle process, as a target of hsa-miR-885-5p.

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