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Original Research Article

TGF-β promotes proliferation and inhibits apoptosis of liver cancer Huh-7 cells by regulating MiR-182/CADM1

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Abstract

Purpose: *To investigate the mechanism of liver cancer cell tolerance to the antiproliferative effect of transforming growth factor beta (TGF-β) based on miRNA levels.*
Methods: *MiRNA microarray and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were used to identify differentially expressed miRNAs in liver cancer Huh-7 cells treated with TGF-β. The effect of these miRNAs on patient survival was analyzed using Kaplan-Meier Plotter. Involvement of Smad2/Smad3 in TGF-β-induced miR-182 expression was determined using shRNA knockdown and qRT-PCR. Dose-dependent effect of TGF-β on miR-182 expression was investigated in Huh-7 cells and mouse primary liver cells using qRT-PCR. The effect of miR-182 on Huh-7 cell proliferation and apoptosis was studied using CFSE and Annexin V/PI assay. Direct targets of miR-182 in Huh-7 cells were identified using a luciferase reporter gene assay, while the influence of Recombinant Cell Adhesion Molecule 1 (CADM1) overexpression on Huh-7 cell proliferation and apoptosis treated with miR-182 was examined using lentivirus experiments, and CFSE and Annexin V/PI assays.*
Results: *The expression levels of hsa-miR-181a, hsa-miR-182, hsa-miR-483, and hsa-miR-143 were significantly higher in serum samples from liver cancer patients ($p < 0.05$). Survival analysis showed that low expression of hsa-miR-182 and high expression of hsa-miR-483 increased the survival rate of liver cancer patients. Furthermore, TGF-β increased miR-182 expression in Huh-7 cells, but not in mouse primary liver cancer cells. The MiR-182 promoted Huh-7 cell proliferation and inhibited apoptosis. It targeted CADM1 mRNA 3'-UTR, decreasing CADM1 expression. Overexpression of MiR-182 significantly reduced cell proliferation and increased apoptosis in Huh-7 cells with CADM1 overexpression ($p < 0.05$).*
Conclusion: *Transforming growth factor beta (TGF-β) facilitates the proliferation and repression of apoptosis of Huh-7 cells by increasing miR-182 expression and inhibiting CADMI expression.*

Keywords: *Transforming growth factor beta (TGF-β), Micro ribonucleic acids (miRNAs), Huh-7 cells, Recombinant Cell Adhesion Molecule 1 (CADM1)*

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INTRODUCTION

Liver cancer, one of the most common cancer-related deaths worldwide [1], had increasing mortality and morbidity rates over the past 20 years despite clinical application of new treatments [2]. It covers such heterogeneous malignant tumors as hepatocellular carcinoma,

intrahepatic cholangiocarcinoma, combined hepatocellular-cholangiocarcinoma, fibrolamellar hepatocellular carcinoma, and hepatoblastoma in children, which have different histological characteristics and poor prognosis. Hepatocellular carcinoma and intrahepatic cholangiocarcinoma are the most common primary liver cancers [3]. Epidemiological studies show that with increasing incidence rate of liver cancer, it is estimated that there may be 1 million cases of liver cancer in the world by 2030 [4]. As a result, there is an urgent need to understand the pathogenesis of liver cancer at molecular level, this is expected to facilitate discovery of new drug targets for the development of drugs for liver cancer.

Transforming growth factor beta (TGF-β), a cell growth inhibitor plays a vital role in the pathological conditions of liver cancer. Besides, TGF-β binds to TGF-bRII (a receptor of TGF-β) to trigger the phosphorylation of TGF-βRI (a receptor of TGF-β), thereby phosphorylation drosophila mothers against decapentaplegic protein2 (Smad2) and Smad3, facilitating cell nuclei entry [5]. Subsequently, Smad2 and Smad3 bind to Smad-binding element (SBE) in gene promoters and regulate the expression of corresponding genes together with different helper transcription factors. TGF-β-induced gene expressions induce the differentiation of liver mother cells into hepatocytes or cholangiocytes in the early stage of liver tissue development [6].

Moreover, studies report that TGF-β represses proliferation and promotes apoptosis of liver cells in normal adult liver tissues. TGF-β impeded the development of tumors in the early stage of liver cancer [7], but it repressed apoptosis and facilitated proliferation of liver cells in advanced stage of liver cancer. Once TGF-β-induces inhibition of proliferation, and promotion of apoptosis of liver cancer cells are surmounted, liver cancer cells will undergo epithelial-mesenchymal transition [8]. It follows that TGF-β plays a crucial role in the pathological process of liver cancer. Furthermore, research on TGF-β inhibitor galunisertib (LY2157299) is now at the clinical stage [9]. However, the mechanism by which liver cancer cells develop tolerance to the antiproliferative and pro-apoptotic effects of TGF-β remains unclear.

Microribonucleic acids (miRNAs) are a class of conservative non-coding RNA molecules with low molecular weight and a length of about 22 nucleotides, that inhibit messenger RNA (mRNA) expressions and modulate gene expressions at the post-transcriptional level. It regulates gene expressions by binding to 3'-untranslated region (3'-UTR) of mRNAs to lead to mRNA degradation or translation inhibition. Numerous studies have revealed that miRNAs play important roles in the pathophysiological process of liver cancer. In this study, TGF-β-induced differentially-expressed miRNAs were investigated to understand the mechanism of liver cancer cells in developing tolerance to the anti-proliferative and pro-apoptotic effects of TGF-β.

EXPERIMENTAL

Cell culture

Liver cancer Huh-7 cells and primary mouse liver cells, obtained from the American Type Culture Collection (ATCC) cell bank in Manassas, VA, USA, were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Invitrogen, Carlsbad, CA, USA; Batch no. 12491015). The culture medium consisted of 10 % horse serum (Invitrogen, Carlsbad, CA, USA; Batch no. 444262) and 5 % fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA; Batch no. 16000-044), and cells were incubated in a temperature-controlled cell incubator with 5 % CO_2 at 37 °C.

Determination of differentially-expressed miRNAs via gene microarray expression profile assay

Total RNAs were extracted from Huh-7 cells using a TRIzol kit (Invitrogen, Carlsbad, CA, USA; Article no. 1903217) and subsequently quantified with a NanoDrop kit (Thermo Fisher Scientific, Waltham, MA, USA). The integrity of the RNAs was evaluated using a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). For this study, 100 ng of total RNAs were utilized along with a 3' IVT Express kit (Affymetrix) to generate complementary RNAs (cRNAs). These cRNAs were then subjected to hybridization on a PrimeView Human microarray (Affymetrix, Santa Clara, CA, USA) at 45 °C for 16 h, following the instructions outlined in the GeneChip 3' Array manual (Affymetrix, Santa Clara, CA, USA). After hybridization, the microarrays underwent processing on an FS-450 fluid station (Affymetrix, Santa Clara, CA, USA) for washing and staining, and were subsequently scanned using a GeneChip scanner (Affymetrix, Santa Clara, CA, USA) in accordance with the manufacturer's guidelines. The raw data contained within the CEL files were imported into the Partek Genomics Suite 6.6 software. Normalization of probe set was performed using Robust Multiarray Average method. The statistical significance of the differentially expressed genes was assessed through a one-way analysis of variance

(ANOVA), with *p*-values corrected using the false discovery rate (FDR) method.

Survival analysis

Online bioinformatics tool Kaplan-Meier Plotter (http://www.kmplot.com/) was utilized to examine the impact of specific miRNA expression variations on patient survival. This analysis aimed to ascertain the influence of miRNA expressions on the survival outcomes of liver cancer patients, ultimately determining the potential biological significance of these miRNAs within the context of liver cancer [10].

Cell protocols

In the initial phase of the study, the impact of TGF-β on miRNA expression profiles in liver cancer Huh-7 cells was assessed. Specifically, Huh-7 cells were subjected to two treatments: 10 ng/mL of TGF-β (experimental group) and 10 ng/mL of lgG (control group), both administered for a 24-hour duration. Subsequently, a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay was conducted using the same experimental conditions as the expression profiling assay. Moving to the subsequent stage, short hairpin RNA (shRNA) targeted against Smad2 (sh-Smad2) or sh-Smad3 was used to knock down protein expression of Smad2 and Smad3. This was accomplished through cell transfection, allowing us to investigate whether the upregulation of miR-182 by TGF-β is contingent on the Smad2/Smad3 signaling pathway. Following a 24-hour treatment with sh-Smad2 or sh-Smad3, cells were exposed to 10 ng/mL of TGF-β for an additional 24 h to examine the impact of TGF-β on miR-182 expression levels. To explore the dose-dependent effect of TGF-β on miR-182 expression, different doses of TGF-β (3, 6, and 12 ng/mL) were administered to Huh-7 cells. Moreover, for comparative purposes, mouse primary cells were employed as a control group to discern if TGF-β induction of miR-182 expression was specific to liver cancer cells. The third phase of the study involved employing flow cytometry to analyze the consequences of miR-182 on Huh-7 cells. This examination was conducted 48 h after transfection of miR-182 mimic (miR-182), miR-182 inhibitor, or miRNA control (miR-con) into Huh-7 cells. Ultimately, the core objective of this study was to investigate whether miR-182 enhances cell proliferation while suppressing apoptosis in Huh-7 cells by downregulating the expression of Cell Adhesion Molecule 1 (CADM1) protein. To bolster CADM1 protein expression, lentivirus experiments were employed to overexpress CADM1. Cells that were successfully transfected for 12 h were subsequently treated with miR-182 for 48 h and subjected to assessment via flow cytometry.

Cell transfection

MiR-182, miR-con, miR-182 inhibitor, sh-CADM1, shRNA control (sh-con), sh-Smad2, and sh-Smad3, along with their corresponding controls, were procured from Shanghai GenePharma Co Ltd. (Shanghai, China). To construct CADM1 recombinant plasmids (GCH1), the complete sequences of CADM1, Smad2, and Smad3 were cloned into pLenti6/V5-D-TOPO vectors (Invitrogen, Carlsbad, CA, USA), with empty plasmids serving as control counterparts. The silencing of CADM1, Smad2, and Smad3 was achieved using shRNA (shRNA, Qiagen, Cambridge, MA, USA). Liver cancer Huh-7 cells were transfected for a duration of 6 h with 30 nM of sh-CADM1, sh-Smad2, and sh-Smad3, utilizing the Lipofectamine 2000TM transfection reagent (Invitrogen, Carlsbad, CA, USA). Following this, the medium containing the transfection reagent was replaced, and the Huh-7 cells were cultured with fresh medium for an additional 48 h. For the transfection of Huh-7 cells with miR-182 mimic and miR-182 inhibitor, Lipofectamine 3000TM transfection reagent (Invitrogen, Carlsbad, CA, USA) was employed, following the manufacturer's instructions provided with the kits. After a successful transfection process, the cells were cultured in fresh medium for a duration of 24 h.

Reverse transcription and quantitative polymerase chain reaction (qRT-PCR) assay

To determine the relevant mRNA expressions in cells, reverse transcription (RT) and qPCR were used. The miRNAs contained within exosomes were subjected to reverse transcription using the PrimeScript RT reagent kit, followed by quantification of miRNA expression using the Taqman quantitative kit (Invitrogen, Carlsbad, CA, USA). In the case of the samples, 500 ng of RNAs were divided into three portions, with each group of total RNAs being diluted tenfold. For PCR amplification, 3 μL of total RNAs was utilized. The amplification of the target genes was verified through 5% agarose gel electrophoresis. Quantification and data processing were conducted using LabWorks 4.0 image acquisition and analysis software. In this study, U6 served as the internal reference. Gene primers were procured from ABM (Peterborough, Camb, Canada). To ensure data reliability, three replicate wells were established for each group of samples. The 2-ΔΔCt method for analysis of changes in relative expression levels of target

genes was used. The primer sequences used in this study are found in Table 1.

Western blotting

An appropriate volume of radioimmunoprecipitation assay (RIPA) solution was mixed with protease inhibitor phenylmethanesulfonyl fluoride (PMSF) at a ratio of 100:1 (RIPA: PMSF) to create the cell lysis buffer (Beyotime, Shanghai, China). Post-trypsinization, cells were collected and treated with the lysis buffer. The resulting mixture was transferred to an Eppendorf (EP) tube and subjected to centrifugation at 4°C and 14000 rpm for 30 min using a low-temperature high-speed centrifuge. This was followed by collecting the protein supernatant. Subsequently, the proteins were denatured by placing the samples in a thermal bath at 95°C for 10 min. The prepared protein samples were then stored at -80°C for future use.

The Pierce bicinchoninic acid (BCA) kit (Rockford, IL, USA) was employed to quantify the protein concentrations. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), gel preparation was carried out, and protein samples were loaded into the gel wells for electrophoresis at a constant voltage of 80 V for 2.5 h. Following protein separation, the samples were transferred to a polyvinylidene fluoride (PVDF) membrane from Roche (Basel, Switzerland) using the semi-dry method. After the transfer, the membrane was immersed in Tris-buffered saline with Tween®20 (TBST) buffer containing 5 % skimmed milk powder. It was gently shaken on a shaker for 1 h to facilitate blocking.

Next, primary antibodies (CADM1 and GAPDH) were diluted with 5% skim milk powder, and the membrane was incubated with these primary antibodies. Afterward, the membrane was rinsed three times with TBST solution (each time for 10 min). The subsequent step involved incubating the membrane with secondary antibody at room temperature for 2 h, followed by two washes with TBST and one wash with TBS (each for 10 min). To detect proteins, electrochemiluminescence (ECL) reagents were employed, and bands were visualized in a dark room. The relative expression levels of the proteins were analyzed using Image-Pro Plus v6 software (Media Cybernetics, Silver Spring, MD, USA).

Luciferase reporter assay

In this experimental setup, the wild-type and mutant CADM1 3'-UTR sequences were amplified and subsequently inserted into psiCHECK-2 luciferase plasmids (Promega, Madison, WI, USA). This process generated wild-type CADM1 and mutant reporter genes. Following this, liver cancer Huh-7 cells were cultivated in a 24-well plate and co-transfected with either miR-182 or miR-con and the wild-type or mutant plasmids. This co-transfection was carried out over 48 h. After the co-transfection duration, the luciferase activity was measured using the dual-luciferase reporter reagent (Promega, Madison, WI, USA). This assay allowed for the assessment of the impact of miR-182 or miR-con on the activity of luciferase driven by either the wild-type or mutant CADM1 3'-UTR sequences.

Determination of cell apoptosis

Alterations in apoptosis among Huh-7 cells were assessed using the Annexin V/propidium iodide (PI) double staining kit. A cell concentration of 5 × 105 cells/mL was achieved through trypsinization, followed by two washes with 4°C phosphate-buffered saline (PBS). Subsequently, centrifugation was carried out.

Table 1: Primer sequences used

Primer name		Primer sequence
CADM1	Forward	5′-GCTTCTGCTGTTGCTCTTCTCC-3′
	Reverse	5′-GACTTGGCAACTGATGGTCGCA-3′
Human serum albumin (hsa)-miR-181a	Forward	5′-AACATTCAACGCTGTCGGTG-3′
	Reverse	5′-GAACATGTCTGCGTATCTC-3′
Hsa-miR-182	Forward	5′-GGCAATGGTAGAACTCAC-3′
	Reverse	5′-GAACATGTCTGCGTATCTC-3′
Hsa-miR-483	Forward	5′-AGACGGGAGGAAAGAAG-3′
	Reverse	5′-GAACATGTCTGCGTATCTC-3′
Hsa-miR-143	Forward	5′-GCAGTGCTGCATCTCTG-3′
	Reverse	5′-GAACATGTCTGCGTATCTC-3′
U6	Forward	5′-TCGACAGTCAGCCGCATCTTCTTT-3′
	Reverse	5′-ACCAAATCCGTTGACTCCGACCTT-3′

The cells were then suspended in 500 µL of staining buffer, after which they were stained with 5 µL of Annexin V-FITC and 5 µL of PI staining solution. This staining process was conducted in a dark environment at a temperature of 37°C, and a duration of 15 min was allotted for the staining to occur. After staining, the cells were loaded into a Guava flow cytometer (FACSCalibur; BD Biosciences, Detroit, MI, USA) for determination and analysis. This technique allowed for the evaluation of apoptosis levels within the Huh-7 cell population.

Determination of cell proliferation via carboxyfluorescein diacetate, succinimidyl ester (CFSE)

Initially, cells were trypsinized, washed three times with phosphate-buffered saline (PBS) (using 4.5 mL of PBS for each wash), and then centrifuged at 500 g for 5 min. They were subsequently resuspended to achieve a concentration of 2 × 10^7 cells/mL. A solution of 2 × 10 µM CFSE was prepared and mixed with the cells in equal proportions, followed by continuous agitation in the dark for 8 min. The staining process was halted by adding an equivalent volume of fetal bovine serum (FBS) and gently blending in the dark at room temperature for 5 min. After centrifuging the mixture at 500 g for 5 min and discarding the supernatant, a mixture of 160 + 10% FBS (4.5 mL) was added to the cells, and after an additional 5 min in the dark, the cells were centrifuged again. Following two washes as described, cells were resuspended in 1640 + 10% FBS medium to attain a final concentration of 1 × 10^6 cells/mL. The cells were then added to a 24-well plate, with each well containing 0.5 mL of the cell suspension. After a 12-hour incubation, the cells underwent a 32-hour treatment period as per the experimental plan. Following treatment, the cells were harvested and prepared for testing. This CFSE-based method enabled the evaluation of cell proliferation dynamics through fluorescent labeling and subsequent analysis.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) 13.0 software (SPSS Inc., Chicago, IL, USA) was used for analysis and t-test for data analysis among different groups. Values are expressed as mean ± standard deviation (SD) and percentage (%). $P < 0.05$ indicate that a difference was statistically significant.

RESULTS

Differentially expressed miRNAs

To determine the effect of TGF-β on liver cancer cells at the epigenetic level, an miRNA microarray expression profile assay was adopted to screen differentially-expressed miRNAs after the liver cancer Huh-7 cells were treated with TGF-β for 24 h. It was observed that with fold change > 1 and $p < 0.05$ as screening criteria, the miRNA expression profiles in cells in TGF-β group were significantly different from those in control group (Figure 1). In comparison with control group, TGF-β group had a total of 16 differentially expressed miRNAs, comprising 5 miRNAs significantly down-regulated and 11 miRNAs overtly up-regulated (Figure 1).

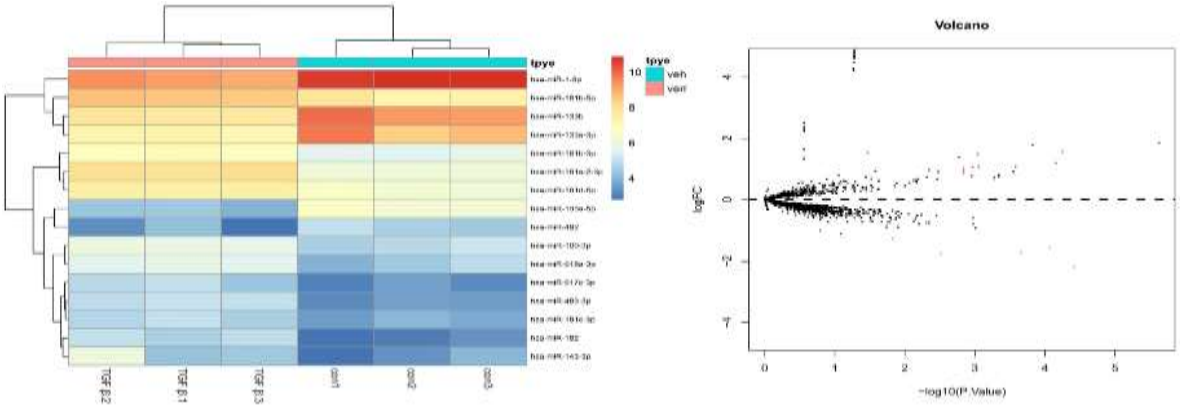

Figure 1: Unsupervised cluster analysis heat map and volcano plot (TGF-β: liver cancer Huh-7 cells treated with TGF-β, con: control cells, each row: one miRNA, red: higher expression content, and blue: a lower expression content). TGF-β-treated liver cancer Huh-7 cells have miRNA expression profiles significantly different from control cells. With fold change > 1 and $p < 0.05$ as screening criteria, there are a total of 16 miRNAs in TGF-β group is significantly different from those in control group, of which 5 miRNAs are down-regulated and 11 miRNAs up-regulated

Differentially expressed miRNAs determined via qRT-PCR

The effects of TGF-β on the expression levels of hsa-miR-181a, hsa-miR-182, hsa-miR-483, and hsa-miR-143 in liver cancer Huh-7 cells were verified *via* RT-qPCR, to confirm the reliability of the high-throughput screening results. Compared with those in control group, the expression levels of hsa-miR-181a, hsa-miR-182, hsa-miR-483, and hsa-miR-143 in Huh-7 cells increased significantly in TGF-β group ($p < 0.01$; Figure 2).

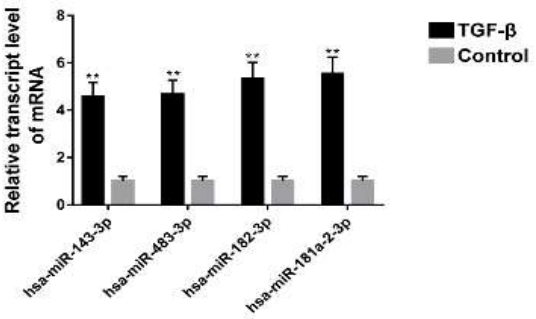

Figure 2: Differentially-expressed miRNAs determined *via* RT-qPCR assay. The expression levels of hsa-miR-181a, hsa-miR-182, hsa-miR-483 and hsa-miR-143 were increased in TGF-β group. ** $p < 0.01$ TGF-β group *vs.* control group

Effects of differentially expressed miRNAs on patient survival time

With Kaplan-Meier Plotter, the influences of hsa-miR-181a, hsa-miR-182, hsa-miR-483, and hsa-miR-143 on the survival time of patients with liver cancer were investigated to study the impacts of mRNAs on patient prognosis. The results revealed that both hsa-miR-182 low-expression group and hsa-miR-483 high-expression group had remarkably improved survival time of patients, while hsa-miR-181a and hsa-miR-143 expression levels had no significant effect on the survival time of patients (Figure 3). Besides, differently expressed hsa-miR-182 had a more significant effect on the survival time of patients, so hsa-miR-182 was selected as the study object for further research in this study.

Smad2 and Smad3 knockdown repressed the induction of TGF-β on miR-182

The miR-182 content was elevated in a dose-dependent manner in Huh-7 cells treated with TGF-β, showing a significant difference, while it did not rise in mouse primary liver cells treated with TGF-β (Figure 4 A). To further explore whether such a TGF-β-induced increase in miR-182 is achieved through the typical TGF-β/Smad signaling pathway, sh-Smad2 or sh-Smad3 was used to knock down the protein expression levels of Smad2 and Smad3 in Huh-7 cells (Figure 4 B). The RT-qPCR assay was carried out, and the results (Figure 4 C) showed that compared TGF-β group, the expression level of miR-182 reduced in TGF-β + Smad2 and TGF-β + Smad3 low-expression groups.

MiR-182 facilitated the proliferation and suppressed the apoptosis of Huh-7 cells

In this study, flow cytometry was employed to determine the effects of miR-182 and miR-182 inhibitors on Huh-7 cells, to determine the effect of miR-182 on the proliferation and apoptosis of Huh-7 cells. The results of CFSE assay (Figure 5) demonstrated that the proliferation rate of Huh-7 cells was clearly higher in miR-182 group and lower in miR-182 inhibitor group than in miR-con group.

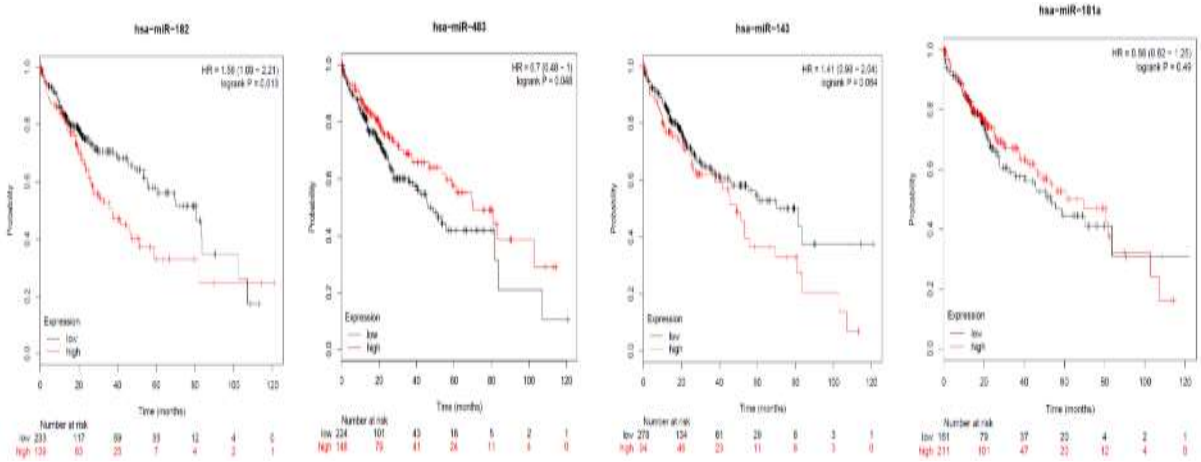

Figure 3: Effects of miRNAs on patient survival time. The survival time of patients is markedly prolonged in hsa-miR-182 low expression group and hsa-miR-483 high expression group

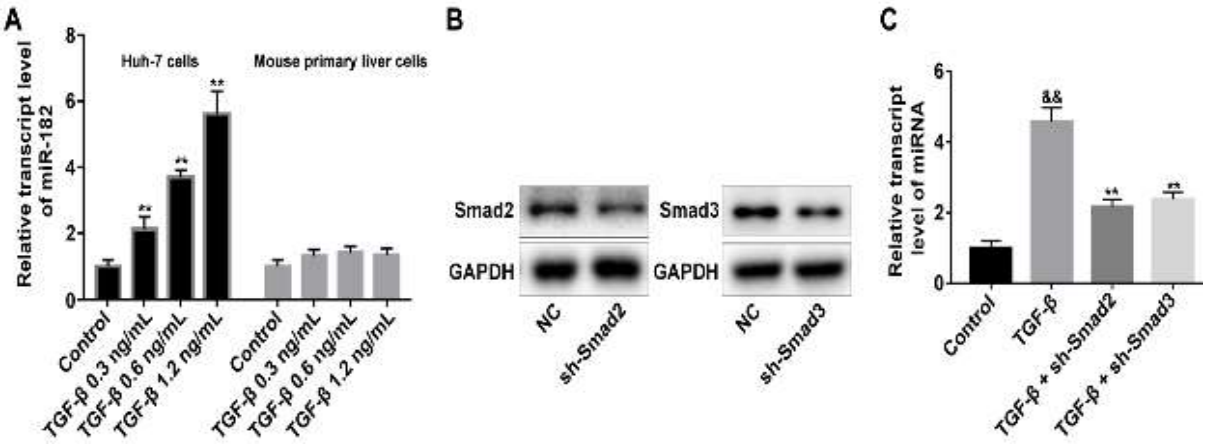

Figure 4: Knockdown of Smad2 and Smad3 inhibit the induction of miR-182 by TGF-β. (A) Effect of different doses of TGF-β on Huh-7 cells and mouse primary liver cells determined by RT-qPCR. (B) Effects of sh-Smad2 or sh-Smad3 on the protein expression levels of Smad2 and Smad3 in Huh-7 cells as determined *via* Western blotting. (C) Effect of sh-Smad2 or sh-Smad3 on the expression level of miR-182 in TGF-β group as assayed through RT-qPCR. $^{**}P < 0.01$ TGF-β group *vs.* control group

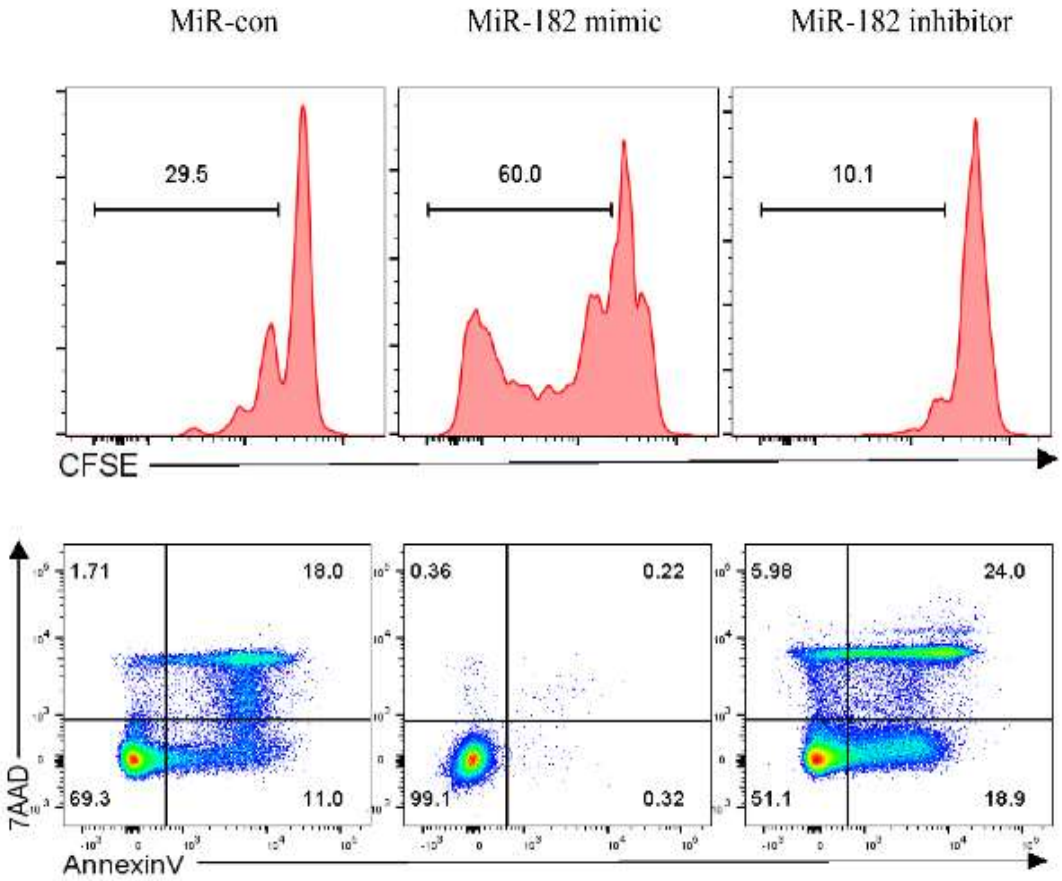

Figure 5: Effects of miR-182 expression level on proliferation and apoptosis of Huh-7 cells. Elevated miR-182 expression level promotes the proliferation of Huh-7 cells and represses their apoptosis. $^{**}P < 0.01$ miR-con group *vs.* miR-182 or miR-182 inhibitor group

Annexin V/PI apoptosis staining results indicated that the apoptosis rate of Huh-7 cells was significantly reduced in miR-182 group, and increased in miR-182 inhibitor group when compared with that in miR-con group.

MiR-182 negatively regulated the expression of CADM1 by targeting the CADM1 mRNA 3'-UTR

Based on biological information predictions, CADM1 might be a potential target of miR-182

(Figure 6 A). To further confirm the interaction between miR-182 and CADM1 gene, the response of wild-type and mutant CADM1 gene to miR-182 and miR-con in Hun-7 cells was determined *via* luciferase reporter assay. The results showed that the intensity of fluorescence response of miR-182-treated Huh-7 cells with wild-type CADM1 gene was significantly reduced, while that of those with mutant CADH1 gene had no obvious changes (Figure 6 B). To obtain further evidence, RT-qPCR and Western blotting assays were performed, and it was found that transfection with miR-182 significantly reduced protein and mRNA levels of CADM1, while, knockdown miR-182 increased the CADM1 gene expression (Figures 6 C, D and E). The aforementioned results suggest that miR-182 binds to the 3'-UTR of CADM1 mRNA, leading to a negative modulation of CADM1 expression.

Overexpressing CADM1 inhibited the promotion of miR-182 on proliferation and its inhibition on apoptosis

To further determine whether miR-182 promotes the proliferation and inhibits the apoptosis of Huh-7 cells by targeting CADM1, the effect of miR-182 on Huh-7 cells was determined after overexpressing CADM1. It was found that compared with those in miR-182 group, the cell proliferation rate was reduced, and the apoptosis rate was increased in miR-182 + CADM1 overexpression group, and the differences were of statistical significance ($p < 0.01$).

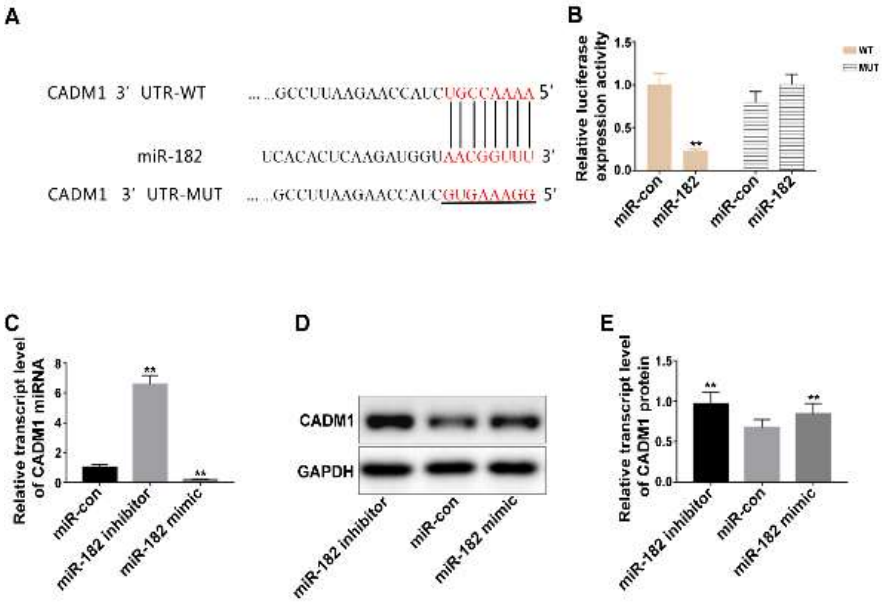

Figure 6: MiR-182 negatively regulates the expression of CADM1 by directly binding to the CADM1 mRNA 3'-UTR. (A) Direct binding sites of miR-182 to the 3'-UTR of CADM1 mRNA predicted using bioinformatics software. (B) Response of wild-type and mutant CADM1 gene to miR-182 and miR-con in Huh-7 determined by luciferase reporter assay. (C, D, and E) Effects of transfection with miR-con, miR-182 and miR-182 inhibitor on the mRNA and protein levels of CADM1 as determined through Western blotting and RT-qPCR. ***P* < 0.01 miR-con group *vs.* miR-182 or miR-182 inhibitor group.

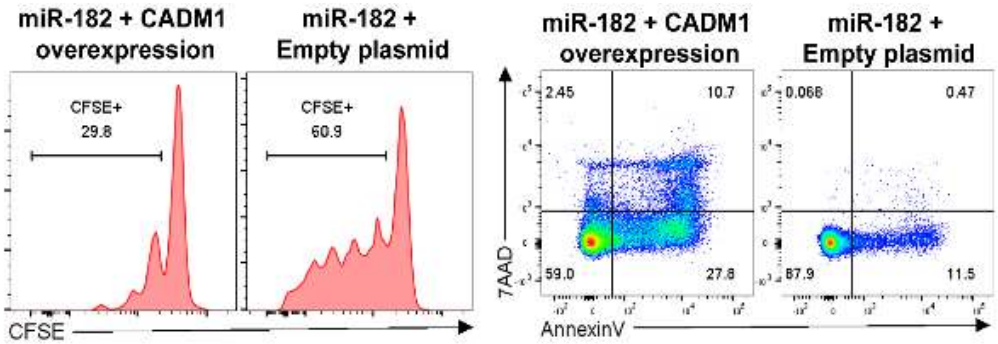

Figure 7: The overexpression of CADM1 hinders the promotion of miR-182 on cell proliferation and its suppressive impact on apoptosis. The cell proliferation rate is remarkably lower in miR-182 + CADM1 overexpression group than in miR-182 group

DISCUSSION

In this study, it was found that TGF-β activated the expression of miR-182 depending on Smad2/Smad3 in Huh-7 cells, while it did not increase the expression level of miR-182 in mouse primary liver cells. Besides, the results of bioinformatics analysis revealed that the survival time of patients in hsa-miR-182 low-expression group was overtly improved, suggesting that hsa-miR-182 may be an important player in the pathological processes of liver cancer. In addition, it was revealed through *in vitro* function verification studies that miR-182 overtly promoted the proliferation and repressed the apoptosis of Huh-7 cells, while miR-182 inhibitor decreased the proliferation rate and increased the apoptosis rate of Huh-7 cells. Moreover, subsequent assays revealed that miR-182 negatively regulated the expression of CADM1 by targeting the CADM1 mRNA 3'-UTR. Furthermore, it was observed in overexpression experiment that overexpression of CADM1 inhibited the proliferation of miR-182 and reversed its inhibitory effect on apoptosis.

Transforming growth factor-beta activates miR-182 expression depending on Smad2/Smad3, while miR-182 targets CADM1 to facilitate proliferation and represses the apoptosis of Huh-7 cells. Furthermore, the results of this study emphasized the vital role of miR-182 in the mechanism of liver cancer cell tolerance to anti-proliferation and pro-apoptosis effects of TGF-β. The TGF-β is an important molecule with anti-proliferative and pro-apoptotic effect. It strictly controls the proliferation of hepatocytes [11] and inhibits tumorigenesis in early stages of liver cancer. However, TGF-β also exert pro-proliferative and anti-apoptotic effect on liver cancer cells in the advanced stage of liver cancer [12]. The resistance of liver cancer cells to inhibition of TGF-β on proliferation has been widely observed, but its possible mechanism of action is still unknown. In this study, it has been reported that TGF-β activated miR-182 expression in a Smad2/Smad3-dependent manner, while it did not elevate miR-182 expression in primary mouse liver cells.

Studies have shown that TGF-β-regulated proliferation-inhibitory phenotypes of liver cancer cells can be induced by mutations in tumor-specific TGF-β receptors and Smad family proteins and changes in methylation or acetylation levels in the promoter region of Smad proteins [13,14]. Moreover, it has been demonstrated that tumor-specific gene mutations lead to a TGF-β-induced increase in the expression of miR-182. There is a shortcoming in this study: a specific mechanism by which TGF-β activates the miR-182 expression in tumor cells was not discovered.

The MiR-182 is a well-known proto-cancer miRNA that promotes proliferation, inhibits apoptosis, and regulates migration and invasion of tumor cells in various tumors [15]. These functions of miR-182 have been reported for liver cancer, and the report is consistent with the findings in this study. For instance, a study conducted by Zhao *et al* [16] showed that miR-182 targets P7TP3 to activate Wnt/β-catenin signaling pathway, promoting tumor development, invasion and migration. In addition, Wang *et al* [17] further reported that LINC01018 inhibited the development and progression of liver cancer by targeting miR-182. Moreover, research by Cao *et al* [18] revealed that miR-182 is conducive to the progression of liver cancer by targeting FOXO3a. It is widely considered that CADM1, also known as TSLC1, acts as a tumor suppressor protein in tumor progression. A lot of research indicates that CADM1 participates in cell-to-cell interaction, and has close correlation with metastasis and invasion of tumors. Besides, another study revealed that CADM1 induces G1/S phase arrest of liver cancer cells through Rb-E2F signaling pathway [19]. Numerous studies report that expression of CADM1 is reduced in many cancers, such as non-small cell lung cancer, breast cancer, cervical cancer and liver cancer, and such decrease is significantly associated with poor prognosis of patients.

CONCLUSION

The findings of this study have shown TGF-β's role in activating miR-182 via Smad2/Smad3 in Huh-7 cells, contrasting with mouse liver cells. Low hsa-miR-182 expression correlates with improved liver cancer patient survival. MiR-182 fuels Huh-7 cell proliferation and curbs apoptosis, targeting CADM1. CADM1 overexpression counters miR-182's effects. This illuminates TGF-β-miR-182-CADM1 axis's role in liver cancer behavior. These findings offer potentials for developing novel therapeutic strategies to modulate this pathway.

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Ethical approval

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Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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