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LncRNA ZEB1-AS1 reduces liver cancer cell proliferation by targeting miR-365a-3p

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Abstract. Liver carcinoma is one of the most common malignancies worldwide. Previous studies have demonstrated that long non-coding RNAs (lncRNAs) are crucial mediators that participate in a wide range of molecular processes associated with carcinogenesis. However, little is known about the specific mechanisms that underlie the majority of lncRNAs. Many studies have indicated that lncRNAs affect microRNA (miRNA or miR) activities via physical base-paired binding, therefore serving as competing endogenous RNAs (ceRNAs) that indirectly regulate the expression of miRNA targets. In the current study, it was revealed that lncRNA zinc-finger E-box binding homeobox 1 antisense 1 (ZEB1-AS1) serves as a ceRNA for miR-365a-3p, functioning to positively modulate E2F transcription factor 2 (E2F2) expression in liver cancer cells. Additionally, reverse transcription-quantitative polymerase chain reaction demonstrated that levels of ZEB1-AS1 were abnormally upregulated in liver cancer and this was positively correlated with E2F2 expression. Furthermore, high levels of ZEB1-AS1 exhibited a trend for poor survival in patients with liver cancer. Western blot analysis demonstrated that ZEB1-AS1 silencing could reduce E2F2 expression. EdU staining and flow cytometry analysis indicated that down-regulation of ZEB1-AS1 could suppress cell proliferation and decrease the S phase proportion of liver cancer cells, which was effectively reversed by the inhibition of miR-365a-3p. ZEB1-AS1 was also determined to be physically associated with miR-365a-3p, while miR-365a-3p was revealed to target the E2F2 3'UTR for degradation or translational repression. The results also demonstrated that ZEB1-AS1 positively regulates E2F2 expression by competitively binding to miR-365a-3p. It was further revealed to enhance liver cancer cell proliferation. Thus, these results indicate that ZEB1-AS1 is required for liver cancer progression in a ceRNA dependent manner. ZEB1-AS1 may therefore be a potential target for liver cancer intervention.

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Introduction

Liver cancer is one of the most frequent lethal malignancies worldwide. In 2012, ~782,500 people were diagnosed with liver cancer and 745,500 mortalities occurred worldwide, with China alone accounting for ~50% of all cases and mortalities (1). Its incidence is also expected to increase in the future (1). Previous studies have advanced the prevention and treatment of liver cancer (2). However, excluding chemotherapy as a conventional option for patients following surgical treatment, other additional interventions, including targeted therapy remain limited (3). It is therefore important to determine the molecular mechanisms underlying the development and progression of liver cancer for the development of potential therapeutic targets.

Long non-coding RNAs (lncRNAs), which are defined as a class of RNA with a length of >200 nucleotides that do not encode proteins (4), have been demonstrated to participate in various cellular and molecular processes, including genomic imprinting, chromatin modification, transcriptional control and post-transcriptional control (5). Given that lncRNAs exert notable functions in various biological processes, the abnormal expression of lncRNAs may therefore be associated with various diseases, including human cancer (6,7). Furthermore, emerging data have revealed that the transcriptome of hepatocarcinoma tissues exhibits a distinct alteration of lncRNAs compared with normal tissues (8-10). However, the majority of these abnormally expressed lncRNAs are poorly investigated and their roles in the development of liver cancer are largely elusive.

Currently, the biological function of microRNAs (miRNAs or miRs) is widely recognized. Mature miRNAs specifically bind to the 3'untranslated region (UTR) of target mRNAs for cleavage or translational inhibition (11). Several lncRNAs have been revealed to function as competing endogenous RNAs

(ceRNAs) that sequester miRNAs via base-pair targeting, which leads to a loss of miRNA function along with the upregulated expression of endogenous target genes (12-14). LncRNAs exhibit a high capacity to modulate the expression of specific targets that are closely associated with tumor cell proliferation, survival, apoptosis, invasion and migration via ceRNA regulation, thus serving crucial roles in cancer initiation and progression (15).

The current study revealed that a particular lncRNA, zinc-finger E-box binding homeobox 1 antisense 1 (ZEB1-AS1), was highly expressed in liver cancer, which may be used to predict a poor prognosis for patients with liver cancer. Furthermore, the results demonstrated that ZEB1-AS1 competitively binds to miR-365a-3p and abolishes the repression on E2F transcription factor 2 (E2F2) caused by miR-365a-3p, resulting in increased E2F2 expression and enhanced liver cancer cell proliferation. The current study enhances the understanding of the mechanism that underlies ZEB1-AS1 in liver cancer progression. Therefore, ZEB1-AS1 maybe a novel target for the therapeutic treatment of liver cancer.

Materials and methods

Clinical samples. The present study was approved by the Ethics Committee of Sichuan Provincial People's Hospital (Chengdu, China) and all patients provided written informed consent. A total of 32 patients (male, n=18 and female, n=14; age range, 49-75 years) diagnosed with hepatocarcinoma who underwent surgery at the Department of Hepatobiliary Surgery, Sichuan Provincial People's Hospital (Chengdu, China) were randomly enrolled between March 2013 and January 2015. Exclusion criteria included radiotherapy or chemotherapy prior to surgical treatment, a prior history of cancer, and a lack of the written informed consent. Hepatocarcinoma tissue and matched adjacent noncancerous tissue samples, 2 cm away from the edge of the carcinoma lesion, were obtained from patients during surgery and experienced pathologists confirmed diagnosis. All procedures were performed in accordance with the Helsinki Declaration.

Cell culture and transfection. HepG2, 293T and HCCLM6 cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare, Chicago, IL, USA) and maintained at 37˚C in a 5% CO_2-humidified incubator. miR-365a-3p mimic (5'-UAAUGCCCCUAAAAAUCCUUAU-3'), mimic negative control (NC; miR-NC; 5'-AUGUACCUAACUAAUCUACACU-3'), miR-365a-3p inhibitor (5'-AUUACGGGGAUUUUUAGGAAUA-3') and an inhibitor negative control (5'-ACUAUUGGAGAUUAGGUAUGUA-3') were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The ZEB1-AS1 short hairpin RNA (shRNA) (5'-GACAGATGTGATCTCTGAACCTGAT-3'), synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China), was cloned into the pGHP1/Neo vector (Shanghai GenePharma Co., Ltd.) to generate sh-ZEB1-AS1, as previously described (16). A scrambled sh-NC (Shanghai GenePharma Co., Ltd.) was used as the negative control. Cells were transfected with 50 nM miRNA mimic, 50 nM miRNA inhibitor or 4.0 μg corresponding plasmids (sh-NC or sh-ZEB1-AS1) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following 48-h transfection, cells were harvested for subsequent experimentation.

To establish stable sh-ZEB1-AS1 cell lines, HepG2 and HCCLM6 cell lines were transfected with sh-ZEB1-AS1 or sh-NC plasmids, respectively, using Lipofectamine® 2000 and stable cell lines were selected for using 800 μg/ml neomycin at 37˚C for ~3 weeks.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells or tissue samples using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using the M-MLV Reverse Transcriptase kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. qPCR was subsequently performed using a SYBR Green Master mix (Takara Biotechnology Co., Ltd., Dalian, China) and the Biosystems Real Time PCR System (Thermo Fisher Scientific, Inc.). The following primer pairs were used for the qPCR: miR-365a-3p forward, 5'-TAATGCCCCTAAAAATCCTTAT-3' and reverse, miScript universal primer (Shanghai GenePharma Co., Ltd.); ZEB1-AS1 forward, 5'-CCGTGGGCACTGCTGAAT-3' and reverse, 5'-CTGCTGGCAAGCGGAACT-3'; E2F2 forward, 5'-GAGCTCACTCAGACCCCAAG-3' and reverse, 5'-AACAGGCTGAAGCCAAAAGA-3'; GAPDH forward, 5'-CTGGGCTACACTGAGCACCAG-3' and reverse, 5'-CCAGCGTCAAAGGTGGAG-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 3 min; 40 cycles of 95˚C for 5 sec and 60˚C for 30 sec. A melt curve step from 65-95˚C was performed in increments of 0.5˚C per 5 sec. Relative mRNA and miRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to GAPDH and U6, respectively (17).

Cell proliferation and cell cycle analysis. Cell proliferation was analyzed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's protocol. Transfected cells were seeded into 96-well plates at a density of $3.0x10^3$ cells/well and cultured for 24, 48, 72 and 96 h, after which 10 μl CCK-8 reagent was added to each well. Cells were incubation for a further 2 h at 37˚C. The optical density of each well was measured at a wavelength of 450 nm using a microplate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each experiment was performed in triplicate.

Proliferating cells were analyzed using the Cell-Light EdU DNA Cell Proliferation kit (Guangzhou Ribobio Co., Ltd., Guangzhou, China), according to the manufacturer's protocol. Proliferating cells were stained with fluorescent-labeled EdU for 2 h at room temperature. For the cell cycle assay, ~$1.0x10^6$ stained cells were fixed using 70% ethanol overnight at 4˚C. Subsequently, cells were stained with propidium iodide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C

for 1 h. Cells were analyzed using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software (version 7.6.1; Tree Star, Inc., Ashland, OR, USA). Each experiment was performed in triplicate (data not shown).

RNA pull-down assay. Biotinylated miR-365a-3p-wild-type (WT) (5'-UAAUGCCCCUAAAAAUCCUUAU-3'), miR-365a-3p-mutant (Mut) (5'-UUUACGGGCUAAAAAUCCUUAU-3') and biotinylated mimic-NC (5'-AUGUACCUAACUAAUCUACACU-3') were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). As previously described (18), HepG2 cells were transfected with the aforementioned biotinylated miRNA mimics (50 nM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following 48-h transfection, cells were harvested and lysed on ice with a buffer containing 200 mM NaCl, 2.5 mM $MgCl_2$, 20 mM Tris, pH 7.5, 0.05% Igepal, 60 U/ml Superase-In (Ambion; Thermo Fisher Scientific, Inc.), 1 mM DTT and protease inhibitors (Roche Applied Science, Rotkreuz, Switzerland) for 10 min. Subsequently, cell lysates were incubated with M-280 streptavidin magnetic beads (Sigma-Aldrich; Merck KGaA) for 3 h at 4˚C. To avoid non-specific binding of protein complexes and RNA, the beads were coated with yeast tRNA and RNase-free bovine serum albumin (Sigma-Aldrich; Merck KGaA). The beads were washed three times with low salt buffer (0.1% SDS, 1% Trition X-100, 2 mM EDTA, 20 mM TrisHCl pH 8.0 and 150 mM NaCl) and once with high salt buffer (0.1% SDS, 1% Trition X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0 and 500 mM NaCl), Following washing steps, bound RNA was extracted using TRIzol® reagent, prior to RT-qPCR analysis, as previously mentioned.

Dual-luciferase reporter assay. Bioinformatic analysis was used to predict target genes of miR-365a-3p using the miRDB database (http://mirdb.org). The WT ZEB1-AS1 cDNA fragment or WT E2F2 3'UTR cDNA containing potential binding sites for miR-365a-3p were amplified by PCR using the PfuUltra II Fusion HS DNA Polymerase (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA). These fragments were subsequently cloned into the pmirGLO vector (Promega Corporation, Madison, WI, USA) and designated pmirGLO-ZEB1-AS1 WT and pmirGLO-E2F2 3'UTR WT, respectively. In addition, the ZEB1-AS1 cDNA or E2F2 3'UTR cDNA fragments with point mutations in the response element of the miR-365a-3p seed sequence region were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) and cloned into the pmirGLO vector and designated pmirGLO-ZEB1-AS1 Mut and pmirGLO-E2F2 3'UTR Mut, respectively. Cells were seeded into 96-well plates at a density of $1.5x10^4$ 293T cells/well and luciferase reporter vectors were co-transfected with miR-365a-3p mimic or miR-NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Following 48-h incubation at 37˚C, cells were collected and the relative firefly and *Renilla* luciferase activities were detected using a Dual-Luciferase Reporter assay system (Promega Corporation), according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Western blotting. Western blotting was performed as previously described (19). Total cellular protein was extracted using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) containing a proteinase inhibitor (Sigma-Aldrich; Merck KGaA). Total protein was quantified using a bicinchoninic acid assay (Beyotime Institute of Biotechnology), following the manufacturer's protocol using a microplate reader (Model 550; Bio-Rad Laboratories, Inc.) In total 10 μg protein/lane was separated via SDS-PAGE on a 10% gel. The separated proteins were transferred onto polyvinylidene difluoride membranes and blocked for 1 h at 25˚C with 5% non-fat milk. The membranes were incubated with primary antibodies against E2F2 (1:500; cat. no. SAB4500684; Sigma-Aldrich; Merck KGaA) and GAPDH (1:1,000; cat. no. 2118; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4˚C. Following primary incubation, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:1,000; cat. no. A0216; Beyotime Institute of Biotechnology) for 2 h at room temperature. Protein bands were visualized using the Pierce ECL Western Blotting kit (Pierce; Thermo Fisher Scientific, Inc.). Protein expression was quantified using Image-Pro® Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. All data are presented as the mean ± standard deviation from three independent experiments. The overall survival was analyzed using the Kaplan-Meier method, and patients were classified as higher or lower than the mean value of ZEB1-AS1 expression. In addition, a Log-rank test was used to analyzed the overall survival data. Pearson's Coefficient was applied to evaluate the correlation between ZEB1-AS1 levels and E2F2 levels. A two-tailed Student's t-test was performed to determine differences between two groups, and paired Student's t-tests were used to determine differences between hepatocarcinoma tissues and matched adjacent noncancerous tissues. $P<0.05$ was considered to indicate a statistically significant difference.

Results

High ZEB1-AS1 expression contributes to liver cancer cell proliferation. Initially, the expression of ZEB1-AS1 was assessed in 32 pairs of hepatocarcinoma tissues and adjacent non-cancerous tissues collected from patients. The results demonstrated that ZEB1-AS1 levels were significantly upregulated in hepatocarcinoma tissues compared with normal adjacent tissues (Fig. 1A). Subsequently, survival analysis was performed on patients. It was revealed that patients with a higher ZEB1-AS1 expression exhibited a significantly poorer overall survival than those with a lower expression (Fig. 1B). These results indicate that ZEB1-AS1 is highly expressed in hepatocarcinoma and may exhibit oncogenic potency. Therefore, the current study aimed to determine the role of ZEB1-AS1 in liver cancer. The expression of ZEB1-AS1 in HepG2 and HCCLM6 cells was knocked down by stably transfecting sh-RNA plasmids (Fig. 1C). Using a CCK-8 assay and EdU staining, it was determined that the depletion of ZEB1-AS1 suppressed cell proliferation and decreased EdU incorporation, indicating that the downregulation of ZEB1-AS1 may inhibit liver cancer cell proliferation (Fig. 1D and E). These

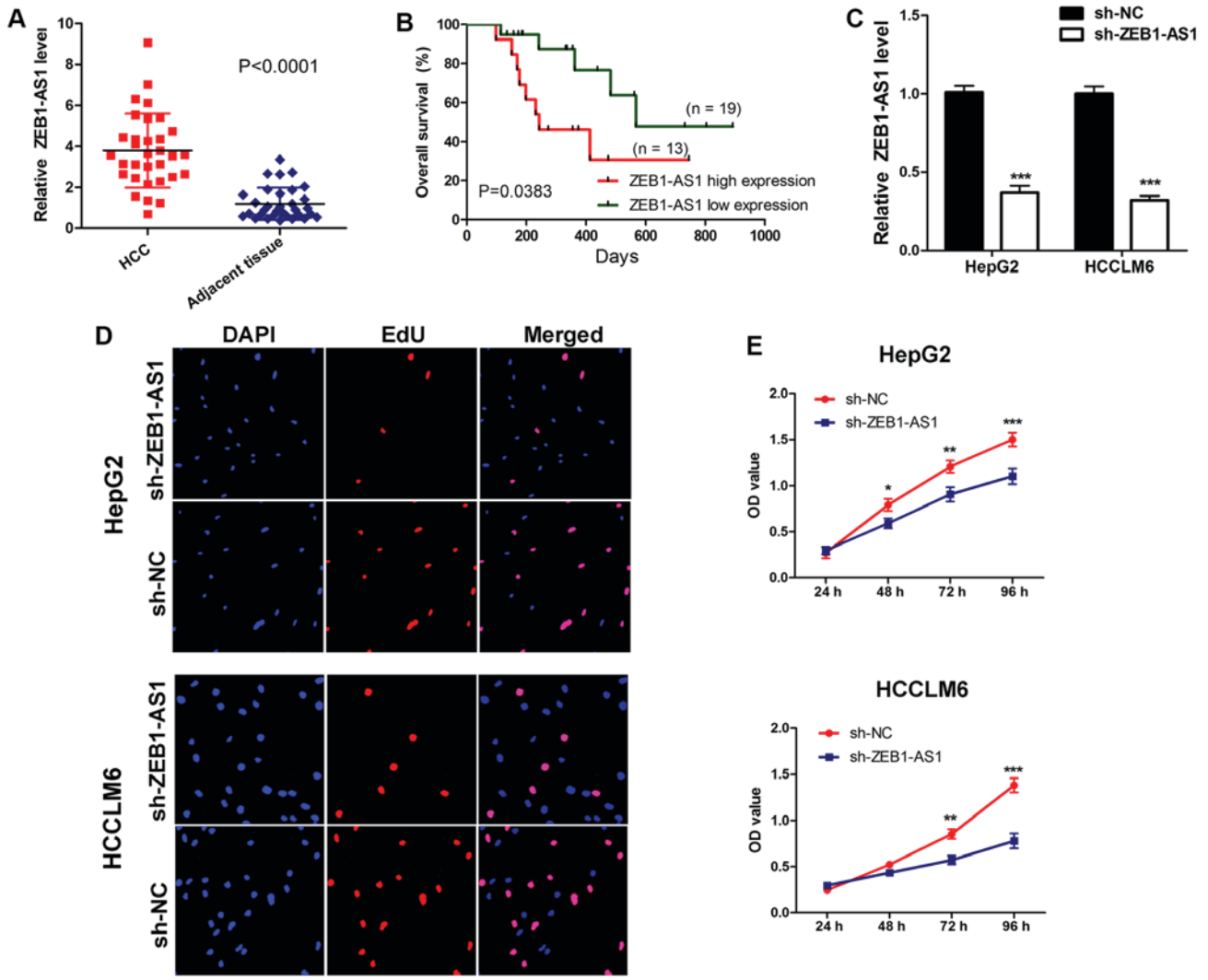


Figure 1. Effect of increased ZEB1-AS1 expression in liver cancer. (A) Relative mRNA level of ZEB1-AS1 was determined in hepatocarcinoma tissue and adjacent normal tissue samples using RT-qPCR. (B) Kaplan-Meier survival analysis curve of 32 patients with hepatocarcinoma classified as high or low ZEB1-AS1 expression based on the population mean value of ZEB1-AS1 expression (3.83). (C) RT-qPCR analysis of ZEB1-AS1 expression in HepG2 and HCCLM6 cells stably transfected with sh-ZEB1-AS1 or sh-NC. (D) EdU staining was utilized to determine cell proliferation. DAPI was utilized to stain nuclei. Magnification, x200. (E) CCK-8 analysis was used to determine cell viability via acell proliferation curve. Data are presented as mean ± standard deviation (n=3). *P<0.05; **P<0.01 and ***P<0.001 vs. sh-NC. ZEB1-AS1, zinc-finger E-box binding homeobox 1 antisense 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; EdU, 5-ethynyl-2'-deoxyuridine; CCK-8, Cell Counting Kit-8; HCC, hepatocarcinoma; NC, negative control; sh, short hairpin.

results imply that ZEB1-AS1 may be required for liver cancer cell proliferation.

ZEB1-AS1 is associated with miR-365a-3p. miRDB target prediction revealed that the sequence of ZEB1-AS1 possessed a response element to the seed sequence of miR-365a-3p, indicating that a direct interaction between ZEB1-AS1 and miR-365a-3p may exist (Fig. 2A). Subsequently, to determine whether miR-365a-3p interacts with ZEB1-AS1 via the seed sequence region, a luciferase reporter assay was performed using reporter vectors containing WT ZEB1-AS1 or Mut ZEB1-AS1 (Fig. 2B). The results demonstrated that the overexpression of miR-365a-3p decreased the luciferase activity of WT reporters but not of empty or Mut reporters (Fig. 2C). To assess whether ZEB1-AS1 directly binds to miR-365a-3p, a biotin-avidin pull-down assay was performed. Biotinylated miR-365a-3p mimics were transfected into HepG2 cells to pull-down associated ZEB1-AS1 (Fig. 2D) and RT-qPCR was subsequently performed to assess ZEB1-AS1 expression. The results demonstrated that ZEB1-AS1 was enriched in the group transfected with WT miR-365a-3p (Bio-miR-365a-3p-wt) but not in the group transfected with Mut miR-365a-3p (Bio-miR-365a-3p-mut; Fig. 2E). These data indicated that miR-365a-3p is able to bind to ZEB1-AS1. The effect of ZEB1-AS1 on the expression of miR-365a-3p was assessed. The results demonstrated that there was no marked difference in the expression of miR-365a-3p in the ZEB1-AS1knockdown and negative control groups (Fig. 2F). Thus, these findings demonstrate that ZEB1-AS1 binds to miR-365a-3p but does not affect the expression of miR-365a-3p. Therefore, ZEB1-AS1 might function as a ceRNA for miR-365a-3p.

miR-365a-3p targets the 3'UTR of E2F2 and negatively regulates the expression of E2F2. Through miRDB database

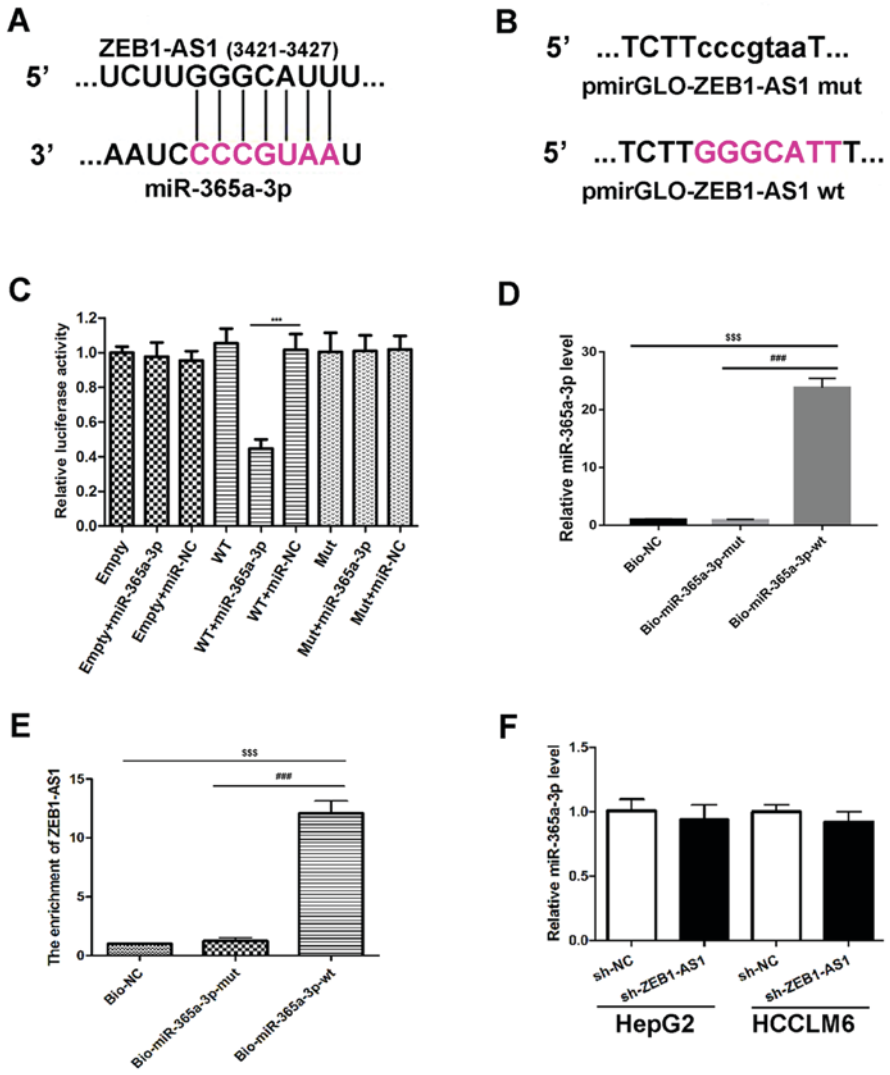


Figure 2. Association between ZEB1-AS1 and miR-365a-3p. (A) Schematic diagram presenting the putative miR-365a-3p recognition site in the ZEB1-AS1 sequence. The miR-365a-3p seed sequence region is marked in pink. (B) Schematic diagram of indicated luciferase reporter vector constructs. The un-mutated bases are highlighted in pink. (C) Luciferase activity of 293T cells co-transfected with miRNA mimics, empty luciferase pmirGLO reporters and luciferase pmirGLO reporters containing ZEB1-AS1 wtor ZEB1-AS1 mut. ***P<0.001 vs. WT+miR-NC. (D) Biotin-based miRNA mimics were transfected into HepG2 cells, followed by RT-qPCR to assess miR-365a-3p expression. (E) RT-qPCR and a biotin-based pull-down assay were utilized to assess ZEB1-AS1 expression in Bio-365a-3p-wt or Bio-365a-3p-mut transfected HepG2 cells. Bio-NC served as the negative control and the relative enrichment of ZEB1-AS1 is presented. $^{\$\$\$}$P<0.001 vs. Bio-NC and $^{\#\#\#}$P<0.001 vs. Bio-miR-365a-3p-mut. (F) RT-qPCR analysis of miR-365a-3p levels in HepG2 and HCCLM6 cells. Cells transfected with sh-NC served as the control. Data are presented as the mean ± standard deviation (n=3). ***P<0.001 vs. WT+miR-NC; $^{\$\$\$}$P<0.001 vs. Bio-NC and $^{\#\#\#}$P<0.001 vs. Bio-miR-365a-3p-mut. ZEB1-AS1, zinc-finger E-box binding homeobox 1 antisense 1; miR, microRNA; WT or wt, wild type; Mut or mut, mutant; Bio, biotinylated; RT-qPCR, reverse transcription quantitative polymerase chain reaction; NC, negative control; sh, short hairpin.

prediction, it was revealed that the E2F2 3'UTR possessed a putative recognition site for miR-365a-3p (Fig. 3A). Therefore, luciferase reporter vectors were generated containing either WT E2F2 3'UTR or Mut E2F2 3'UTR which lacked the response element to the seed sequence region of miR-365a-3p (Fig. 3B). Subsequently, luciferase plasmids were co-transfected with miR-365a-3p mimics or miR-NC into 293T cells. The results demonstrated that the luciferase activity of co-transfectedmiR-365a-3p mimics and WT E2F2 3'UTR was decreased compared with the controls, indicating that miR-365a-3p targets the E2F2 3'UTR at the seed sequence region (Fig. 3C). Furthermore, RT-qPCR demonstrated that transfecting miR-365a-3p mimics without miR-NC led to reduced E2F2 mRNA expression in HepG2 and HCCLM6 cells (Fig. 3D and E). In addition, western blotting results were consistent with RT-qPCR data, indicating that the overexpression of miR-365a-3p resulted in the downregulation of E2F2 protein (Fig. 3F). These results indicate that miR-365a-3p targets E2F2 3'UTR and inhibits E2F2 expression.

ZEB1-AS1 positively modulates E2F2 expression. Given that ZEB1-AS1 was demonstrated to bind to miR-365a-3p without affecting the expression of miR-365a-3p, the current study subsequently assessed whether ZEB1-AS1 indirectly affected the target gene of miR-365a-3p by serving as a ceRNA. The results demonstrated that ZEB1-AS1knockdown induced a significant decrease of E2F2 mRNA in HepG2 and HCCLM6 cells (Fig. 4A). Additionally, depleting ZEB1-AS1 also reduced E2F2 protein expression in liver cancer cells (Fig. 4B). Subsequently, the expression of E2F2 mRNA in

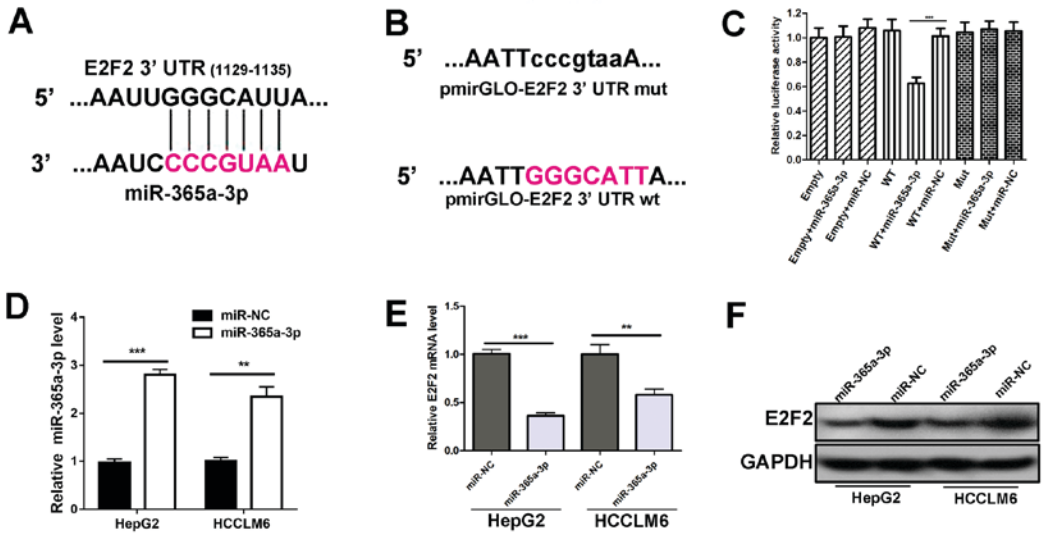


Figure 3. Regulation of E2F2 by miR-365a-3p. (A) Schematic diagram presenting the putative miR-365a-3p response element on the E2F2 3'UTR. The miR-365a-3p seed sequence region is marked in pink. (B) Schematic diagram of the luciferase reporter vector constructs utilized. (C) Luciferase activity in 293T cells co-transfected miRNA mimics, empty luciferase reporter pmirGLOs and luciferase reporter pmirGLOs containing E2F2 3' UTR WT or E2F2 3'UTR Mut. ***P<0.001 vs. WT+miR-NC. (D) RT-qPCR analysis of miR-365a-3p levels in HepG2 and HCCLM6 cells transfected with miR-365a-3p mimics or miR-NC. **P<0.01 and ***P<0.001 vs. miR-NC. (E) RT-qPCR analysis of E2F2 mRNA levels in HepG2 and HCCLM6 cells transfected with miR-365a-3p mimics or miR-NC. **P<0.01 and ***P<0.001 vs. miR-NC. (F) Western blot analysis of E2F2 in HepG2 and HCCLM6 cells transfected with miR-365a-3p mimics or miR-NC. Data are presented as the mean ± standard deviation (n=3 miR or miRNA, microRNA; UTR, untranslated region; WT, wild type; mut, mutant; NC, negative control; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

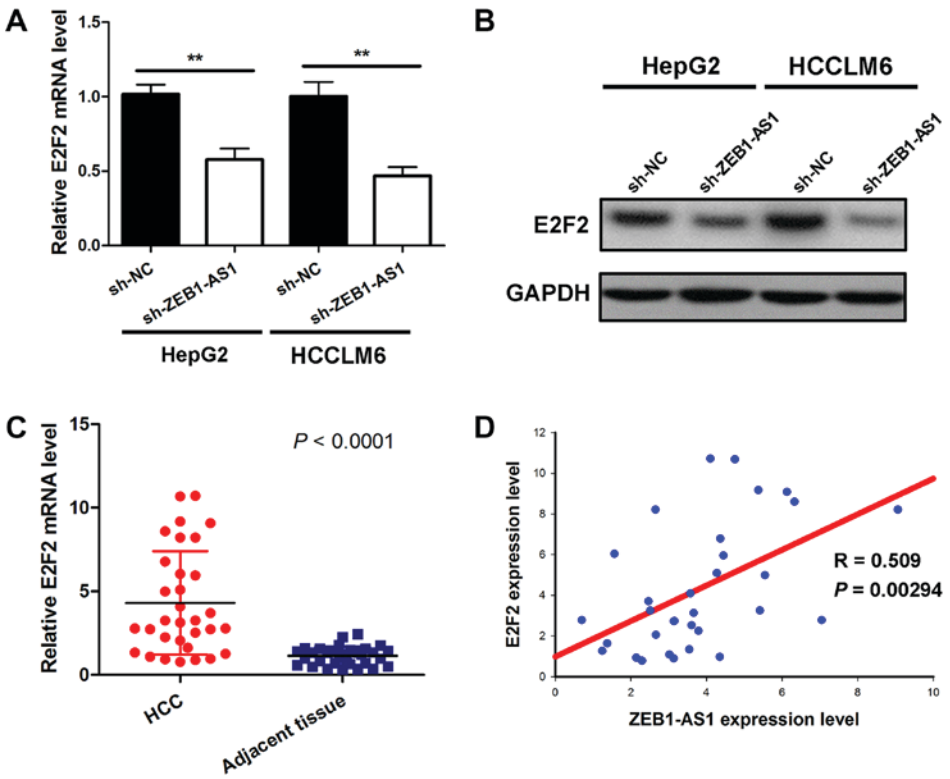


Figure 4. Regulation of E2F2 by ZEB1-AS1. (A) RT-qPCR analysis of E2F2 mRNA levels in HepG2 and HCCLM6 cells transfected with indicated plasmids. (B) E2F2 expression in HepG2 and HCCLM6 cells was determined via western blotting (C) RT-qPCR analysis of E2F2 mRNA levels in 32 pairs of hepatocarcinoma tissue and matched adjacent normal tissue samples. (D) Pearson's correlation analysis of ZEB1-AS1 and E2F2 mRNA levels in 32 hepatocarcinoma tissue samples. Data are expressed as the mean ± standard deviation (n=3). **P<0.01 vs. sh-NC. ZEB1-AS1, zinc-finger E-box binding homeobox 1 antisense 1; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; sh, short hairpin; NC, negative control; HCC, hepatocarcinoma.

hepatocarcinoma and matched para-carcinoma tissues from 32 patients was assessed. The results demonstrated that E2F2 levels in tumor tissues were significantly higher than those in adjacent normal tissues (Fig. 4C). A correlation analysis was

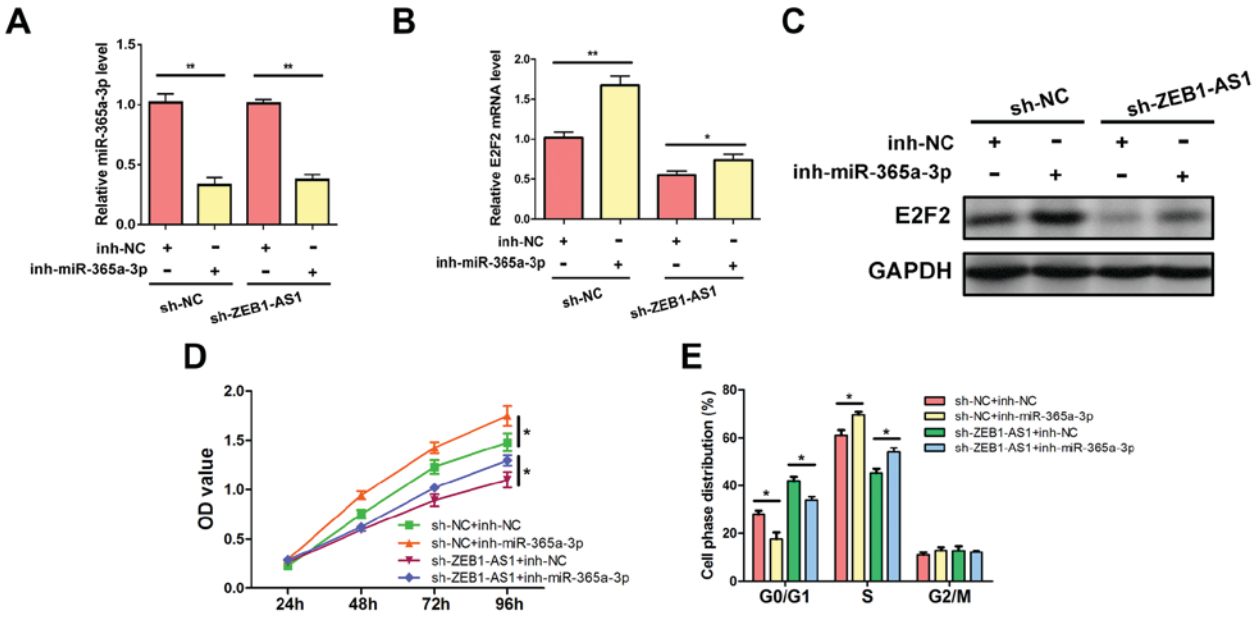


Figure 5. Regulation of E2F2 expression and cell proliferation via ZEB1-AS1 and miR-365a-3p. (A) RT-qPCR analysis of miR-365a-3p levels in HepG2 cells co-transfected with indicated plasmids and miRNA inhibitors. (B) RT-qPCR analysis of E2F2 mRNA levels in HepG2 cells co-transfected with indicated plasmids and miRNA inhibitors. (C) Western blot analysis of E2F2 expression in HepG2 cells co-transfected with indicated plasmids and miRNA inhibitors. (D) Cell Counting Kit-8 analysis of HepG2 cell viabilities presented as a cell proliferation curve. (E) Flow cytometric analysis of HepG2 cell cycle distribution. All data are presented as the mean ± standard deviation. *P<0.05 and **P<0.01. ZEB1-AS1, zinc-finger E-box binding homeobox 1 antisense 1; miR and miRNA, microRNA; inh, inhibitor; NC, negative control; sh, short hairpin.

then performed on ZEB1-AS1 and E2F2 levels. The results demonstrated that ZEB1-AS1 levels were positively correlated with E2F2 levels in hepatocarcinoma tissues (Fig. 4D). Thus, these results support the hypothesis that ZEB1-AS1 positively regulates E2F2 expression in liver cancer.

ZEB1-AS1 regulates E2F2 expression and liver cancer cell proliferation by targeting miR-365a-3p. To ascertain whether ZEB1-AS1 modulates E2F2 expression by affecting miR-365a-3p repression activity, rescue experiments were performed in which the effects of ZEB1-AS1silencing were reversed by the inhibition of miR-365a-3p. The expression of miR-365a-3p was suppressed by the miR-365a-3p inhibitor (Fig. 5A). The results revealed that the inhibition of miR-365a-3p abrogated the decreased expression of E2F2 mRNA and protein, which was caused by ZEB1-AS1silencing in HepG2 cells (Figs. 5B and C). The associated phenotypes were also examined. The knockdown of ZEB1-AS1 suppressed HepG2 cell viability; however, the rescue of miR-365a-3p inhibition abolished this effect and re-elevated cell proliferation (Fig. 5D). In addition, flow cytometric analysis demonstrated that the downregulation of ZEB1-AS1 resulted in the decrease of HepG2 S phase populations and increased G0/G1 period proportions, which were also abrogated by the inhibition of miR-365a-3p (Fig. 5E). These results demonstrated that ZEB1-AS1 competitively binds to miR-365a-3p and inhibits the repressive effect of miR-365a-3p on E2F2, which contributes to elevated E2F2 expression and enhanced cell proliferation in liver cancer.

Discussion

The ZEB1-AS1 transcript is a non-coding antisense RNA that originates from the ZEB1 promoter region, which was initially reported in hepatocellular cancer (20). Previous studies revealed that ZEB1-AS1 is located in physical contiguity with ZEB1 and that it positively modulates ZEB1, induces epithelial to mesenchymal transition (EMT) and drives cancer metastasis (20,21). Previous studies have also demonstrated that ZEB1-AS1 functions as an oncogene and promotes the progression of certain tumors including bladder cancer, osteosarcoma, glioma and esophageal squamous cell carcinoma (16,22,23). However, the exact mechanism underlying the oncogenic role of ZEB1-AS1 has not yet been fully elucidated. In the present study, consistent with a previous report, ZEB1-AS1 was highly expressed in liver cancer tissue samples (21). In addition, the high level of ZEB1-AS1 may be a predictor of poor prognosis in patients with liver cancer. The present study also revealed that ZEB1-AS1 exerted oncogenic effects *in vitro* by enhancing liver cancer cell proliferation and promoting cell cycle progression.

Previous studies have primarily elucidated the mechanism by which ZEB1-AS1 exhibits tumor-promoting effects by serving as a crucial regulator of ZEB1 expression (16,21,24). Currently, three mechanisms by which ZEB1-AS1 controls the expression of ZEB1 have been proposed. The first suggests that ZEB1-AS1 may function as an enhancer and thus positively regulates ZEB1 promoter activity (21). In the second theory, ZEB1-AS1 directly binds and recruits p300 to the ZEB1 promoter region, activating ZEB1 transcription (16). Thirdly, ZEB1-AS1 may serve as a molecular sponge for miR-101 to relieve the inhibition of ZEB1 caused by miR-101 (24). Increased ZEB1-AS1 expression has been demonstrated to contribute to the upregulated expression of ZEB1, the progression to EMT and cancer metastasis (20). Furthermore it is also significantly associated with tumor progression and patient survival, in many different types of cancer (25). However,

few studies have examined other mechanisms that involve the oncogenic role of ZEB1-AS1. Given the complexity of tumor genetics, there may exist other pathways and mechanisms by which ZEB1-AS1 contributes to tumor progression.

In the present study, the ceRNA role of ZEB1-AS1 in liver cancer progression was assessed. The results revealed that ZEB1-AS1 interacts with miR-365a-3p and inhibits miR-365a-3p function. Many previous studies have also demonstrated that miR-365 functions as a tumor-suppressor in various types of cancer (26,27). A previous study also revealed that the overexpression of miR-365 suppressed hepatocellular carcinoma growth and induced apoptosis by directly targeting Bcl-2 (19). The results of the current study demonstrated that ZEB1-AS1 serves as a ceRNA for miR-365a-3p to positively regulate the expression of E2F2, which is a target of miR-365a-3p. It was determined that ZEB1-AS1 levels were positively correlated with E2F2 expression in liver cancer tissues, which indicated a potential positive regulation between ZEB1-AS1 and E2F2 transcripts. Furthermore, bioinformatics analysis indicated that ZEB1-AS1 and E2F2 3'UTR shared identical miR-365a-3p recognition sites. These results indicated that ZEB1-AS1 is physically associated with miR-365a-3p, while miR-365a-3p targets E2F2 3'UTR for the degradation of RNA or the inhibition of translation. Additionally, ZEB1-AS1 silencing led to reduced E2F2 mRNA and protein levels, which was abolished by the inhibition of miR-365a-3p. This may be due to ZEB1-AS1 competitively binding to miR-365a-3p, causing a loss of miR-365a-3p function in the repression of E2F2. Although depleting ZEB1-AS1 exhibited little effect on total miR-365a-3p expression, low ZEB1-AS1 might allow for increased levels of free miR-365a-3p, reducing the level of E2F2 expression. Thus, miR-365a-3p inhibition may increase the level of E2F2 expression.

The mammalian E2F family of transcription factors (E2Fs) regulate a variety of cellular functions associated with cell proliferation, differentiation, apoptosis and cell cycle progression (28). Among these family members, E2F2, as a transcriptional activator of E2F target genes, exerts critical effects on the regulation of G1/S transition as well as DNA replication in mammalian cells (29). Increasing evidences have revealed that E2F2 functions as a tumor activator in gastric, breast and non-small cell lung cancer (30-32). Furthermore, it has been revealed that E2F2 is a tumor-promoter in liver cancer (33). The current study demonstrated that the ZEB1-AS1/miR-365a-3p axis serves an important role in the regulation of E2F2 expression as well as in liver cancer cell proliferation and cell cycle progression, indicating that targeting this axis maybe a novel approach for the efficacious intervention of liver cancer.

However, there are limitations in the current study. The expression of ZEB1-AS1 and E2F2 were analyzed, and survival analysis was performed using limited sample sizes. In addition, corresponding knockout experiments were not performed. Thus, further studies are required to validate the expression of ZEB1-AS1 and E2F2 in a large sample size and to further assess their correlation and clinical significance. Knockout experiments should also be considered in future studies to elucidate the role of ZEB1-AS1 in liver cancer *in vivo* or *in vitro*.

Based on the results of the current study, ZEB1-AS1 was determined to positively modulate E2F2 expression and enhance cell proliferation by competitively binding tomiR-365a-3p. The current study further elucidated the mechanism underlying ZEB1-AS1 in liver cancer progression. The results demonstrated that ZEB1-AS1 maybe a potential target for liver cancer therapy.

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Availability of data and materials

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

Authors' contributions

LM and GX designed the study. LM and GH analyzed the data and prepared the figures. GX and LY prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Sichuan Provincial People's Hospital (Chengdu, China).

Patient consent for publication

All the patients provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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