Long Non-Coding RNA ZEB2-AS1 Promotes Hepatocellular Carcinoma Progression by Regulating The miR-582-5p/FOXC1 Axis

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Abstract

Objective: Long non-coding RNAs (lncRNAs) feature prominently in tumors. Reportedly, lncRNA zinc finger E-box-binding homeobox 2 antisense RNA 1 (ZEB2-AS1) is aberrantly expressed in a variety of tumors. The present study was aimed to explore ZEB2-AS1 functions and determine mechanism in hepatocellular carcinoma (HCC) progression.

Materials and Methods: In this experimental study, expressions of ZEB2-AS1, microRNA (miR)-582-5p and forkhead box C1 (FOXC1) mRNA in HCC tissues and cell lines were detected via quantitative reverse transcription polymerase chain reaction (qRT-PCR). After establishing gain- and loss-of-function models, cell counting kit-8, 5-bromo-2'-deoxyuridine (BrdU), Transwell assays and flow cytometry analysis were conducted to examine HCC cell multiplication, migration, invasion and apoptosis, respectively. The targeted relationship between miR-582-5p and ZEB2-AS1 was verified via dual-luciferase reporter gene assay. Western blot was utilized for detecting FOXC1 expression in HCC cells after selectively regulating ZEB2-AS1 and miR-582-5p.

Results: In HCC tissues and cells, ZEB2-AS1 expression was increased. High ZEB2-AS1 expression was related to relatively large tumor volume, increased tumor-node-metastasis (TNM) stage and positive lymph node metastasis of the patients. ZEB2-AS1 overexpression facilitated HCC cell multiplication, migration, invasion and suppressed apoptosis, while ZEB2-AS1 knock-down caused the opposite effects. It was also confirmed that ZEB2-AS1 could competitively bind with miR-582-5p to repress its expression, and indirectly up-regulate FOXC1 expression level in HCC cells.

Conclusion: The current study revealed that ZEB2-AS1 was over-expressed in HCC tissues and cells. It also up-regulated FOXC1, through sponging miR-582-5p, to promote HCC progression. This provides new perspectives for elucidating the pathogenesis of HCC.

Keywords: Forkhead Box C1, Hepatocellular Carcinoma, Long Non-Coding RNA, miR-582-5p

Introduction

Globally, known to rank third among the causes of cancer-related deaths, hepatocellular carcinoma (HCC) makes up about 90% of primary liver cancer cases (1, 2), and half of deaths occurred in China (3). Hepatitis C or B virus (HCV or HBV) infection is one of the primary risk factors for HCC tumorigenesis (4). Despite the recent improvements in treatments, such as liver transplantation, hepatectomy, radiotherapy, chemotherapy and targeted therapy, five-year overall survival rate of HCC patients is still very low as a result of metastasis and recurrence (5, 6).

Recognized as a kind of non-coding RNA, long non-coding RNAs (lncRNAs) are with limited or without protein coding ability. They consist of over 200 nucleotides in length (7). lncRNAs regulate diverse biological processes, for instance, cell differentiation, proliferation, embryonic development and tumorigenesis (8, 9). A great deal of research has shown that lncRNAs feature prominently in cancer biology, regulating tumor cell proliferation, drug resistance and epithelial-mesenchymal transition (EMT) (10-12). Previous studies proved that lncRNA zinc finger E-box-binding homeobox 2 antisense RNA 1 (ZEB2-AS1) was aberrantly expressed in several tumors and was strongly associated with tumorigenesis and cancer progression. For example, ZEB2-AS1 facilitated colorectal carcinoma cell multiplication and repressed apoptosis by enhancing β-catenin protein expression (13). Down-regulated ZEB2-AS1 expression suppressed HCC cell multiplication and metastasis via modulating ZEB2 expression (14). However, the mechanism of ZEB2-AS1 underlying HCC progression needs in-depth investigation.

Known as a type of single-stranded small non-coding RNAs, microRNAs (miRNAs or miRs) bind to mRNA 3′-untranslated region (3′-UTR) to negatively modulate gene expression, inducing the degradation of targeted messenger RNAs (mRNAs) (15). Reportedly, miR-582-5p functions as a tumor suppressor in different cancers.
For example, in bladder cancer, miR-582-5p represses cell multiplication via reducing human monopolar spindle 1 (HMPS1/TTK) expression (16). miR-582-5p was lowly expressed in HCC and it repressed cell multiplication by targeting CDK1 and AKT3 (17). Nevertheless, miR-582-5p molecular mechanism in HCC needs to be further investigated.

The forkhead box (FOX) transcriptional factor family shared a winged helix-turn-helix DNA binding domain and this domain is crucial in regulating cell differentiation, metabolism, proliferation, migration, invasion and apoptosis (18-20). Reportedly, overexpression of forkhead box C1 (FOXO1) induced transactivation of CXCR1 and CCL2 and facilitated HCC cell migration and invasion (21).

In the current research, we reported that ZEB2-AS1 was up-regulated in HCC cell lines and tissues.

Materials and Methods

Tissue samples

Endorsed by the Research Ethics Committee of Wuhan Brain Hospital (Wuhan, China, Approval No. 2019-0517), this study was performed. All patients’ informed consent was obtained and the present study enrolled 50 HCC patients who admitted to the hospital. The clinicopathological data of all patients were obtained and none of them underwent chemotherapy or radiotherapy before the surgery. The cancerous and the corresponding adjacent tissues were surgically removed and collected. Additionally, the cancer tissues of 20 breast cancer patients were obtained from our hospital, and then all the tissues were preserved at -196°C in liquid nitrogen.

Cell culture and transfection

In this experimental study, HCC cell lines (BEL7402, HCCLM3, SMMC-7721 and Huh7) and normal liver cell line (MIHA) were obtained from China Center for Type Culture Collection (Wuhan, China). From the American Type Culture Collection (Manassas, USA), we bought human breast cancer cell line MCF-7. These cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, USA) containing 10% fetal bovine serum (FBS), 100 µg/ml streptomycin and 100 U/ml penicillin (ThermoFisher Scientific, USA) at 37°C in 5% CO2.

Small interfering RNA (siRNA) against ZEB2-AS1 (si-ZEB2-AS1-1, si-ZEB2-AS1-2 and si-ZEB2-AS1-3), siRNA control (si-NC), miR-582-5p inhibitors (miR-582-5p-in), inhibitors control (miR-in), miR-582-5p mimics (miR-582-5p), mimics control (miR-NC), ZEB2-AS1 overexpression vector (ZEB2-AS1) and empty vector (Vector) were synthesized by RiboBio (Guangzhou, China). The oligonucleotides and plasmids were transfected into HCC cells using Lipofectamine 3000 (Invitrogen).

Quantitative reverse transcription polymerase chain reaction

TRIzol reagent (Vazyme, China) was utilized for total RNA isolation. PrimeScript RT reagent kit (TaKaRa, China) was applied for complementary DNA (cDNA) synthesis. For miRNAs, the PrimeScript miRNA cDNA Synthesis Kit (TaKaRa) was adopted to carry out reverse transcription. SYBR Premix Ex Taq I was employed to conduct qRT-PCR. GAPDH and U6 acted as internal references for mRNA and miRNA, respectively. The 2-ΔΔCt method was applied for calculation of the relative expression level. The primers used were as follows:

**ZEB2-AS1-**

F: 5′-GGCTGGATAGCAAAGGAC-3′
R: 5′-ACAATCTTGCGAGGT-3′

**miR-582-5p-**

F: 5′-GCACACATTGAGAGGACAGAC-3′
R: 5′-TATGAGGGGTTCTGGTG-3′

**FOX1-**

F: 5′-CAGAACACGATCCGCACA-3′
R: 5′-TGTTATGGAGTCCGTC-3′

**U6-**

F: 5′-GCTTCGGCAGCACATATAAAT-3′
R: 5′-CGCTTCAGAATTGCGTCT-3′

**GAPDH-**

F: 5′-CACCACACTCCTACACATTTG-3′
R: 5′-CCACACACTCGCTGCTTAG-3′

Cell counting kit-8 (CCK-8) assay

CCK-8 assay was conducted to evaluate cell proliferation. HCC cells were inoculated at 1×10³ cells/well in 96-well plates and they were cultured at 37°C in 5% CO₂ for 24 hours. After culturing cells for 0, 1, 2, 3 and 4 days, 10 µl of CCK-8 reagent (Beyotime Biotechnology, China) was added to each well, followed by cell culture at 37°C in 5% CO₂ for another 2 hours. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad, USA). Four days later, cell proliferation curve was drawn.

5-Bromo-2-deoxyUridine (BrdU) assay

The BrdU method was used to determine DNA synthesis in proliferating cells. BrdU assay was conducted 48 hours after transfection. Briefly, the cells were inoculated in 96-well plates (2×10³ cells/well) and cultured for 48 hours. Subsequently, the cells were incubated with a final concentration of 10 µM BrdU solution (Wuhan AmyJet Scientific Inc., China) for 4 hours at room temperature, followed by medium removal after the incubation period. The cells were fixed for 30 minutes with paraformaldehyde and then incubated with anti-BrdU antibody (Sigma-Aldrich, China) for 1 hour at room temperature in dark. After that, they were washed with PBS three times and...
subsequently incubated with DAPI staining solution at room temperature for 30 minutes in dark. Eventually, the cells were observed under a fluorescence microscope.

Transwell assay

For migration assay, the upper Transwell compartment (BD Biosciences, USA) was loaded with 1x10^5 cells (in 200 μl of serum-free DMEM). Then, the lower one was loaded with 700 μl of 10% FBS-containing DMEM. 24 hours later, cells on the membrane underside were stained and then they were counted in three randomly selected high-power fields, under the microscope. For invasion assay, the chambers were precoated with a layer of Matrigel and the same procedures were conducted as described above.

Flow cytometry assay

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Vazyme, China) was adopted to evaluate the apoptosis of HCCLM3 and BEL7402 cells. The cells were harvested 48 hours after transfection and the cell concentration was adjusted to 1x10^6 cells/ml with binding buffer. Subsequently, the cells were incubated overnight with pre-cooled 70% ethanol at 4˚C. After being centrifuged, the cells were re-suspended in binding buffer (200 μl). The re-suspended cells were then stained with PI staining solution (5 μl) and Annexin V-FITC staining solution (10 μl) in dark for 15 minutes at room temperature. Afterwards, a MoFlo XDP flow cytometer (Beckman Coulter, USA) was utilized to analyze apoptotic cells. Data were processed by BD FACSDiva™ software (BD Bioscience, USA).

Xenograft model in nude mice

Animal Care and Use Committee of Wuhan Brain Hospital approved all animal experiments. Male BALB/c nude mice were bought from Experimental Animal Center of Wuhan University (Wuhan, China). Twenty nude mice (age: 5 weeks) were randomly divided into two groups, with 10 mice in each group. In the lung metastasis experiment, HCCLM3 cells (2x10^6 cells per mouse), overexpressing ZEB2-AS1, or the control cells were injected into the tail vein of nude mice. The mice were euthanized after 3 weeks and the lungs tissues were collected for pathological examination. Lung metastasis was evaluated by hematoxylin-eosin (HE) staining. In brief, after fixation at room temperature for 4 hours, using 4% paraformaldehyde, the lung tissues were dehydrated with ethanol and embedded in paraffin blocks. Then, the tissues were sliced and lung tissue sections with a thickness of 5 μm were dewaxed in xylene, rehydrated in ethanol of decreasing concentrations and washed with PBS. Then, HE staining solution (Beyotime, China) was employed to stain the sections for 5 minutes at room temperature. Next, the sections were dehydrated, sealed and observed under a light microscope.

Western blotting

To obtain protein samples, RIPA lysis buffer was employed for lysing the cells and the supernatant was collected after centrifugation. A bicinchoninic acid kit (Abcam, USA) was utilized for determining the concentration of each protein sample. Next, equivalent amounts of proteins were dissolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to the polyvinyliden fluoride membrane (Millipore, USA), which were subsequently blocked with 5% skimmed milk. Then, the membranes were incubated with anti-FOXC1 antibody (1:1000; Abcam, USA) and anti-GAPDH antibody (1:1000; Abcam, USA) for 12 hours at 4˚C. Then, the membranes, after washing, were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000; Abcam, USA) for 2 hours in room temperature. Subsequently, the enhanced chemiluminescence reagent (Beyotime, China) was employed for developing the protein bands and a ChemiDoc MP system (Bio-Rad, USA) was utilized for the visualization.

Dual-luciferase reporter gene assay

To generate the wild-type (WT) luciferase reporter vector (ZEB2-ASI-WT and FOXC1-WT), the sequences of ZEB2-ASI or FOXC1 3’-UTR containing the binding site for miR-582-5p were integrated into the pmirGLO luciferase reporter vector (Promega, USA). Meanwhile, the binding sites were mutated to produce the corresponding mutant (MUT) luciferase reporter vectors (ZEB2-ASI-MUT and FOXC1-MUT). The constructed vectors and miR-582-5p or miR-NC were co-transfected into HCCLM3 and BEL7402 cells. Ultimately, 48 hours after transfection, the luciferase reporter detection system (Promega) was utilized to analyze the luciferase activity of the cells.

Statistical treatment

All experiments were conducted in triplicate. All data in the experiments were analyzed by GraphPad Prism 8.0 software (GraphPad Software Inc., USA) and SPSS 22.0 (SPSS Inc., USA). A Chi-square test ($\chi^2$ test) was employed for analyzing the relationship between clinicopathological features and ZEB2-ASI expression in HCC samples. The measurement data were expressed as mean ± standard deviation (mean ± SD). Comparison of data among the multiple groups was performed by one-way analysis of variance, and a t test was performed for the comparison between two groups. P<0.05 denoted that a difference was of statistical significance.

Results

ZEB2-ASI was highly expressed in HCC and it was related to the patient’s poor prognosis

To assess ZEB2-ASI expression, qRT-PCR was conducted in normal tissues (n=50), HCC tissues (n=50) and breast cancer tissues (n=20). It was indicated that ZEB2-ASI expression was markedly up-regulated in the HCC tissues against the normal tissues and breast cancer.
ZEB2-AS1 and Hepatocellular Carcinoma

tissues (Fig.1A). Besides, qRT-PCR was conducted to detect ZEB2-AS1 expression in the normal liver cell line (MIHA cells), HCC cell lines (SMMC-7721, BEL7402, HCCLM3 and HuH7 cells) and breast cancer cell line, MCF-7. It was unveiled that, in contrast to the MIHA and MCF-7 cells, ZEB2-AS1 expression in the four types of HCC cell was up-regulated (Fig.1B). Subsequently, the GEPIA database was employed for analyzing the relationship between HCC patients overall survival time and ZEB2-AS1 expression, and it was discovered that in comparison with HCC patients with low ZEB2-AS1 expression, those with the high expression had a lower overall survival rate (Fig.1C). Moreover, the correlation between HCC patient clinicopathological indicators and ZEB2-AS1 expression was analyzed by chi-square test, and the results suggested that high ZEB2-AS1 expression was linked to relatively large tumor volume, increased tumor-node-metastasis (TNM) stage and positive lymph node metastasis (Table 1). The aforementioned evidences implied that ZEB2-AS1 played a cancer-promoting role in HCC.

**Table 1:** Correlation of ZEB2-AS1 expression with multiple clinicopathological characteristics in hepatocellular carcinoma (HCC) patients (n=50)

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**ZEB2-AS1** facilitated HCC cell proliferation, migration, invasion and inhibited cell apoptosis

To probe into **ZEB2-AS1** role in HCC progression, we selected HCCLM3 cells with the lowest **ZEB2-AS1** expression and BEL7402 cells with the highest expression to construct **ZEB2-AS1** overexpression or knock-down models, respectively (Fig.2A). Then, CCK-8 and BrdU assays were conducted to detect HCC cell multiplication. Against the control group, overexpression of **ZEB2-AS1** remarkably promoted HCCLM3 cell proliferation, while **ZEB2-AS1** knock-down notably repressed BEL7402 cell proliferation (Fig.2B-D). Additionally, Transwell assays showed that **ZEB2-AS1** overexpression significantly facilitated HCCLM3 cell migration and invasion, while knocking-down **ZEB2-AS1** inhibited BEL7402 cell migration and invasion (Fig.2E-H). Flow cytometry analysis revealed that overexpression of **ZEB2-AS1** suppressed HCCLM3 cell apoptosis and **ZEB2-AS1** knock-down increased BEL7402 cell apoptosis (Fig.2I, J). The results of HE staining revealed that as opposed to the Vector/HCCML3 group, there were significantly more metastasis nodules in the mouse lung tissues of the **ZEB2-AS1**/HCCML3 group (Fig.S1, See Supplementary Online Information www.celljournal.org). The above results indicated that **ZEB2-AS1** facilitated HCC growth and metastasis.

**Fig.2:** Effects of **ZEB2-AS1** on HCC cell proliferation, migration, invasion and apoptosis. **A.** Detection by qRT-PCR of **ZEB2-AS1** expression in HCCLM3 cells transfected with **ZEB2-AS1** overexpression plasmids and BEL7402 cells transfected with **ZEB2-AS1** siRNAs. **B-D.** CCK-8 and BrdU assays (scale bars: 75 μm) were conducted for detecting HCC cell proliferation after **ZEB2-AS1** overexpression or knock-down. **E-H.** Transwell assay was used to detect HCC cell migration and invasion (scale bars: 250 μm). **I, J.** Flow cytometry was conducted to evaluate apoptosis rate of HCCLM3 and BEL7402 cells after overexpression or knock-down of **ZEB2-AS1**. All experiments were repeated 3 times, each in triplicate. **∗∗∗;** P<0.001, **∗∗;** P<0.01, **∗;** P<0.05. HCC; Hepatocellular carcinoma, qRT-PCR; Quantitative reverse transcription PCR, siRNA; Small interfering RNA, CCK-8; Cell counting kit-8, and BrdU; 5-Bromo-2-deoxyUridine.
**ZEB2-AS1 directly targeted miR-582-5p**

To decipher mechanism of ZEB2-AS1 in HCC progression, bioinformatics was adopted for predicting miRNAs pairing with ZEB2-AS1 and it was uncovered that there existed potential binding sites between ZEB2-AS1 and miR-582-5p (Fig.3A). Dual-luciferase reporter gene assay validated that miR-582-5p mimics could markedly reduce ZEB2-AS1-WT luciferase activity, but exerted no remarkable impact on ZEB2-AS1-MUT luciferase activity (Fig.3B). Subsequently, qRT-PCR displayed that ZEB2-AS1 overexpression significantly inhibited miR-582-5p expression, while ZEB2-AS1 knockdown up-regulated miR-582-5p expression (Fig.3C). Additionally, qRT-PCR was conducted for evaluating miR-582-5p expression in 50 cases of HCC and para-cancerous tissues, and it was discovered that miR-582-5p expression was dramatically down-regulated in HCC tissues in comparison with adjacent normal tissues (Fig.3D). At the same time, we observed that miR-582-5p and ZEB2-AS1 expressions in HCC tissues were inversely related (Fig.3E). The aforementioned evidences suggested that ZEB2-AS1 directly targets miR-582-5p and negatively regulates its expression in HCC.

**miR-582-5p reversed the impact of ZEB2-AS1 on HCC cell multiplication, migration, and apoptosis**

To dig deeper into the role of the ZEB2-AS1/miR-582-5p axis in HCC, we transfected ZEB2-AS1 overexpression plasmid, miR-582-5p mimic, ZEB2-AS1 overexpression plasmid+miR-582-5p into HCCLM3 cells, respectively and transfected si-ZEB2-AS1-1, miR-582-5p inhibitors, si-ZEB2-AS1-1+miR-582-5p inhibitor into BEL7402 cells, respectively. Furthermore, HCC cell multiplication, migration and invasion were detected through CCK-8, BrdU, Transwell assays and flow cytometry analysis. The results manifested that, as opposed to the control group, ZEB2-AS1 overexpression markedly facilitated HCC cell proliferation, migration, invasion and inhibited cell apoptosis, while miR-582-5p mimics suppressed HCC cell proliferation, migration, invasion and increased cell apoptosis, in addition to weakening the effects of ZEB2-AS1 overexpression on HCC cells; additionally, knocking-down ZEB2-AS1 dramatically restrained HCC cell proliferation, migration, invasion and promoted cell apoptosis, while miR-582-5p inhibitors facilitated HCC cell proliferation, migration and invasion. It also suppressed cell apoptosis and partially counteracted the inhibiting effects of si-ZEB2-AS1-1 on the malignant phenotypes of HCC cells (Fig.4A-E).

![Fig.3: miR-582-5p is the target of ZEB2-AS1 in HCC cells. A, Bioinformatics was adopted for predicting binding site between ZEB2-AS1 and miR-582-5p. B, Binding relationship between miR-582-5p and ZEB2-AS1 in HCC cells was detected by dual-luciferase reporter gene assay. C, Detection via qRT-PCR of miR-582-5p expression in HCC cells with overexpression or knockdown of ZEB2-AS1. D, Detection via qRT-PCR of miR-582-5p expression in 50 cases of HCC tissues and adjacent normal tissues. E, Detection of the correlation between ZEB2-AS1 and miR-582-5p expressions in HCC tissues via qRT-PCR. All experiments were repeated 3 times, each in triplicate. **; P<0.01, ***; P<0.001. HCC; Hepatocellular carcinoma, and qRT-PCR, Quantitative reverse transcription polymerase chain reaction.](image)

![Fig.4: miR-582-5p reverses effects of ZEB2-AS1 on HCC cell proliferation, migration, invasion and apoptosis. ZEB2-AS1 overexpression plasmid, miR-582-5p mimic, ZEB2-AS1 overexpression plasmid+miR-582-5p were transfected into HCCLM3 cells, respectively, and si-ZEB2-AS1-1, miR-582-5p inhibitors, si-ZEB2-AS1-1+miR-582-5p inhibitor were transfected into BEL7402 cells, respectively. A, B: CCK-8 and BrdU assays were utilized for examining HCCLM3 and BEL7402 cell proliferation. C, D: HCCLM3 and BEL7402 cell migration and invasion were detected through Transwell assays. E: Flow cytometry analysis was utilized to detect HCCLM3 and BEL7402 cell apoptosis. All experiments were repeated 3 times, each in triplicate. *; P<0.05, **; P<0.01, ***; P<0.001. HCC; Hepatocellular carcinoma, qRT-PCR; Quantitative reverse transcription polymerase chain reaction, CCK-8; Cell counting kit-8, and BrdU; 5-Bromo-2-deoxyUridine.](image)
binding site between miR-582-5p and FOXC1 (Fig. 5A). Subsequently, results of the dual-luciferase reporter gene assay manifested that miR-582-5p mimics could significantly inhibit FOXC1-WT luciferase activity, but failed to change the luciferase activity of FOXC1-MUT (Fig. 5B, C). qRT-PCR displayed that FOXC1 expression was increased in HCC tissues and cells (Fig. 5D, E). Additionally, ZEB2-AS1 overexpression enhanced FOXC1 expression in HCCLM3 cells, while miR-582-5p mimics could down-regulate FOXC1 expression and weaken the promoting effect of ZEB2-AS1 overexpression on FOXC1 expression (Fig. 5F, G). On the other hand, knock-down of ZEB2-AS1 could inhibit FOXC1 expression, while miR-582-5p inhibitors could promote FOXC1 expression and partially reverse the effect of si-ZEB2-AS1-1 on FOXC1 expression (Fig. 5H, I). Moreover, in HCC tissues, miR-582-5p and FOXC1 mRNA expressions were inversely related, while ZEB2-AS1 and FOXC1 mRNA expressions were positively correlated (Fig. 5J, K). The above-mentioned results suggested that ZEB2-AS1 played a role in HCC by regulating the miR-582-5p/FOXC1 axis.

Discussion

In recent years, growing evidences showed lncRNAs are strongly associated with the tumorigenesis and progression of a variety of malignancies. Thus, it can be used as specific markers for certain tumors, and feature prominently in regulating cancer cell proliferation and metastasis. For example, lncRNA LOC284454 promoted HCC cell invasion and migration by suppressing E-cadherin expression (23). Reportedly, ZEB2-AS1 expression was increased in lung cancer tissues. It is suggested that ZEB2-AS1 can facilitate lung cancer cell multiplication and inhibit apoptosis (24). Depletion of ZEB2-AS1 expression suppressed HCC cell growth and metastasis. In the current study, cancer-promoting role of ZEB2-AS1 in HCC was also confirmed, which is in line with the previous study (14). It was manifested that ZEB2-AS1 expression was increased in HCC tissues and cells. High ZEB2-AS1 expression was related to unfavorable clinicopathologic characteristics. What is more, ZEB2-AS1 overexpression remarkably boosted HCC cell multiplication, migration, invasion and suppressed cell apoptosis, while ZEB2-AS1 knock-down had the opposite effects. The aforementioned evidences demonstrated that ZEB2-AS1 could act as a potential HCC prognostic biomarker and therapeutic target.

miRNAs participate in regulating carcinogenesis and cancer progression (25). It was reported that miR-582-5p was aberrantly expressed in various cancers and it could play a cancer-suppressing role. For example, miR-582-5p inhibited bone metastasis of prostate cancer cells by inhibiting TGF-β signal transduction (26). The up-regulation of miR-582-5p repressed endometrial cancer cell multiplication and promoted apoptosis by targeting AKT3 (27). This study unveiled that miR-582-5p expression was underexpressed in HCC tissues. Moreover, miR-582-5p overexpression notably repressed cell multiplication, migration, invasion and promoted cell apoptosis, while miR-582-5p inhibition had the opposite effects. These suggested that miR-582-5p inhibited HCC progression as a tumor suppressor.

lncRNAs can directly interact with miRNAs and act as competing endogenous RNAs (ceRNAs) to modulate miRNA expression. For example, ZEB2-AS1 up-regulated HMGB1 expression via sponging miR-204 to promote the growth and invasion of pancreatic cancer cells (28). ZEB2-AS1 boosted laryngeal squamous cell cancer development via modulating miR-6840-3p/PLXNB1 axis (29). In this study, we found, through bioinformatics analysis, that there existed binding sites between miR-582-5p and ZEB2-AS1. Dual-luciferase reporter gene assay validated that ZEB2-AS1 could sponge miR-582-5p. Additionally, miR-582-5p could weaken the effect of ZEB2-AS1 on HCC cell multiplication, migration, invasion and apoptosis. Therefore, we concluded that ZEB2-AS1 participated in facilitating HCC cell multiplication, migration, invasion and suppressing apoptosis via targeting miR-582-5p.
FOXC1 gene is located on chromosome 6p25, and it functions as a transcription factor (30). The FOX family partakes in various biological processes, for instance, embryonic development, cell cycle regulation, metabolic control, stem cell niche maintenance and signal transduction (31). FOXC1 takes part in the progression of various tumors, and highly expressed FOXC1 is strongly associated with the cancer patient poor prognosis (32). FOXC1 expression was increased in lung cancer and FOXC1 facilitated lung cancer cell proliferation and invasion, and its high expression was related to the low survival rate of lung cancer patients (33). In colorectal cancer, FOXC1 contributed to chemoresistance and it facilitated tumor growth by regulating the miR-31-5p/LATS2 pathway (34). A previous study showed that dysregulation of miR-582-5p/FOXC1 axis could inhibit migration and invasion of salivary adenoid cystic cancer cells (22). In this study, with the TargetScan database, it was revealed that there existed a binding site between miR-585-5p and FOXC1 3’ UTR. It was further validated by dual-luciferase reporter gene assay that miR-582-5p could bind to FOXC1 3’-UTR. Furthermore, overexpression of ZEB2-AS1 or knock-down of miR-582-5p was able to remarkably up-regulate FOXC1 expression. Therefore, we concluded that ZEB2-AS1 was able to participate in regulating HCC progression via targeting miR-582-5p/FOXC1 axis. Our work also partly explained the mechanism of FOXC1 dysregulation in HCC.

Conclusion

Collectively, ZEB2-AS1 promoted HCC cell proliferation, migration and invasion via modulating the miR-582-5p/FOXC1 axis. Our study helps elucidate the mechanism underlying HCC development, in addition to presenting potential treatment targets and biomarkers for HCC.

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Authors’ Contributions

SM.W., J.C.; Conceived and designed the experiments. Y.L., YY.T.; Performed the experiments. Q.L.; Supervised the experiments. L.X.; Analysed the data. J.C.; Wrote the manuscript. J.C.; R.E.; Read and approved the final manuscript. All authors have read and approved the final manuscript.

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