

LncRNA FOXD2-AS1 facilitates the progression of hepatocellular carcinoma by regulating TWIST1

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Abstract. – OBJECTIVE: The study aimed at elucidating the role of FOXD2-AS1 in facilitating the malignant progression of hepatocellular carcinoma (HCC) by regulating TWIST1.

PATIENTS AND METHODS: Relative levels of FOXD2-AS1 and TWIST1 in HCC tissues classified by tumor size and tumor node metastasis (TNM) staging were detected by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The Kaplan-Meier method was applied to assess the prognostic potential of FOXD2-AS1 in HCC patients, followed by survival rate comparison using a log-rank test. After the knockdown of FOXD2-AS1 in HepG2 cells, the viability and migratory abilities were examined by Cell Counting Kit-8 (CCK-8) and transwell assay, respectively. The subcellular distribution of FOXD2-AS1 was detected. Finally, the involvement of TWIST1 in the regulation of HCC cell functions influenced by FOXD2-AS1 was explored.

RESULTS: FOXD2-AS1 was upregulated in HCC tissues, especially large tumor size or stage III-IV cases. High levels of FOXD2-AS1 predicted poor prognosis in HCC patients. FOXD2-AS1 was mainly distributed in the nucleus, and knockdown of FOXD2-AS1 weakened proliferative and migratory abilities in HepG2 cells. TWIST1 was upregulated in HCC tissues, which was positively correlated to FOXD2-AS1 level. The overexpression of TWIST1 could reverse the inhibited proliferative and migratory abilities in HepG2 cells with FOXD2-AS1 knockdown.

CONCLUSIONS: FOXD2-AS1 facilitates the progression of HCC by upregulating TWIST1.

Key Words:

Hepatocellular carcinoma, FOXD2-AS1, TWIST1.

Introduction

Hepatocellular carcinoma (HCC) is a commonly detected tumor, which is the fourth leading cause of cancer death¹. HCC treatment is

difficult because of intrahepatic and extrahepatic metastases². It is of significance to clarify the mechanism underlying HCC metastasis, which is conducive to developing drugs against HCC metastasis.

Long non-coding RNAs (lncRNAs) are non-coding RNAs exceeding 200 bp in length. They are vital transcriptional and post-transcriptional regulators by interacting with DNAs, RNAs and proteins³. So far, multiple lncRNAs have been detected to be abnormally expressed in HCC samples, which are related to HCC progression and prognosis, such as UCA1⁴, MALAT-1⁵ and PVT1⁶. FOXD2-AS1 is located on human chromosome 1p33 with 2509 bp long. It contains an exon and cannot encode proteins. FOXD2-AS1 was initially detected in HCC samples⁷. With the in-depth studies, FOXD2-AS1 has been found to be upregulated in many types of tumors. It drives tumor progression by regulating tumor cell functions^{8,9}. Lei et al¹⁰ reported that FOXD2-AS1 silences DKK1 and activates the Wnt signaling by binding EZH2, thus triggering HCC progression.

TWIST is located on human chromosome 7p21.2, containing 2 exons and 1 intron. The first exon of TWIST is 772 bp long, and the intron is 538 bp long. The TWIST family contains TWIST1 and TWIST2, and the former subtype is highly conserved, which belongs to the Basic/Helix-Loop-Helix transcription factor family^{11,12}. Yang et al¹³ confirmed that the knockdown of TWIST1 remarkably inhibits lung metastases of breast cancer. Li et al¹⁴ pointed out that CASC15 induces epithelial-mesenchymal transition (EMT) and carcinogenesis of HCC by enhancing TWIST1 activity. In this paper, we aim to explore the biological functions of FOXD2-AS1 and TWIST1 in HCC progression and their influences on HCC prognosis.

Patients and Methods

Baseline Characteristics

Eighty primary HCC patients admitted in Guizhou Provincial People's Hospital from January 2017 to June 2017 were recruited. They were treated by surgery and postoperatively confirmed as HCC. Diagnosis was based on the Clinical Guidelines for Hepatocellular Carcinoma (2010) released by American Association for the Study of Liver Diseases (AASLD). Briefly, imaging examinations, such as ultrasound, enhanced CT scanning, and enhanced MRI confirmed the characteristic liver cancer. Typical manifestations included a significantly enhanced lesion in the arterial phase, which was not evident in the venous phase than surrounding liver tissues, and continuously disappeared in the delayed phase; and/or pathological confirmation by liver biopsy or surgical resection¹⁵. None of them had preoperative anti-HCC treatment. During the surgery, HCC tissues and para-tumor ones (≥ 2 cm from the edge of cancer tissues) were collected and stored at -80°C within 15 min of resection. This study was approved by the Ethics Committee of Guizhou Provincial People's Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

HCC cell line HepG2 and normal hepatocytes LO2 were cultivated in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). Cell passage was conducted in 0.25% trypsin containing EDTA (ethylenediaminetetraacetic acid).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

RNAs were extracted from cells using TRIzol (15596018; Thermo Fisher, Waltham, MA, USA), and reversely transcribed to complementary deoxyribose nucleic acids (cDNAs) for qRT-PCR, performed with an ABI7500 Thermal Cycler (Applied Biosystem, Australia), using the SYBR green qPCR kit (AORT-0020, QP001; Genecopoeia, Rockville, MD, USA). Primer sequences were as follows: FOXD2-AS1 F: 5'-TGTTTCGTGGGAAGAGGGTTG-3', R: 5'-TACCACTCCGGAACTCTGT-3'; TWIST1 F: 5'-GGCTCAGCTACGCCTTCTC-3', R: 5'-TCCTTCTCTGGAAACAATGACA-3'; glyc-

eraldehyde 3-phosphate dehydrogenase (GAPDH) F: 5'-ACTGCCACCCAGA-AGACT-3', R: 5'-GCTCAGTG-TAGCCCAGGAT-3'.

Cell Transfection

Cells were implanted in 6-well plates with 2×10^5 cells per well. Until cells were grown to 70% confluence, they were transfected using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced at 6-8 h. At 48 h, medium containing 2 $\mu\text{g}/\text{mL}$ puromycin was applied for 72 h cell culture. Afterwards, the cells were implanted into another 6-well plate for 1-2 weeks of cell culture. Cell colonies were selected for extended culture. FOXD2-AS1 siRNA sequences were as follows: siRNA-FOXD2-AS1 1#: 5'-GCGCGGUUGUUGAGACCAAGG-3' (sense) and 5'-UUGGUCUCAACAACCGCG-CAG-3' (antisense); siRNA-FOXD2-AS1 2#: 5'-GCGAAGAGUACGUUGCUAUTT-3' (sense) and 5'-AUAGCAACGUACUCUUCGCTT-3' (antisense); siRNA-FOXD2-AS1 3#: 5'-GGC-UUUUCCACUAGUUACUGA-3' (sense) and 5'-AGUAACUAGUGGAAAAGCCCA-3' (antisense).

Cell Counting Kit-8 (CCK-8)

Cells were inoculated in a 96-well plate with 5×10^3 cells per well, and six replicates were set. At day 0, 1, 2 and 3, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was applied per well for 1 h incubation and optical density at 450 nm was measured using Infinite M200 Pro (TECAN, Switzerland).

Transwell Assay

Cells were collected and suspended in a serum-free medium. 10 μL of serum-free suspension containing 1×10^4 cells and 500 μL of complete medium were respectively applied in the top and bottom chamber. After 24 h cell culture, transwell chambers were taken out. The cells in the bottom were subjected to methanol fixation for 15 min, and crystal violet staining for 20 min. Images were observed at $40\times$ magnification using an optical microscope (Nikon, Japan) and migratory cells were counted in 10 randomly selected fields per sample.

Subcellular Fraction Determination

Cytoplasmic and nuclear RNAs were extracted using the Cytoplasmic & Nuclear RNA Purification Kit (21000, Norgen, Belmont, CA, USA) and subjected to qRT-PCR using an ABI7500

Thermal Cycler (Applied Biosystem, Australia). U6 was the internal reference of the nucleus and GAPDH was that of cytoplasm.

Statistical Analysis

Statistical analyses were conducted using Statistical Product and Service Solutions (SPSS) 22.0 (IBM Corp., Armonk, NY, USA). The differences between groups were compared using the Student's *t*-test. The Kaplan-Meier method was applied for survival analysis, followed by survival rate comparison using a log-rank test. Correlation between levels of FOXD2-AS1 and TWIST1 in HCC tissues was analyzed by Pearson correlation test. $p < 0.05$ was considered as statistically significant.

Results

Poor Prognosis in HCC Patients Expressing High Level of FOXD2-AS1

Compared with para-tumor tissues, FOXD2-AS1 was upregulated in HCC tissues (Figure 1A).

Notably, FOXD2-AS1 level was higher in HCC tissues with large tumor size (>5 cm) than those smaller ones (Figure 1B). In addition, higher abundance of FOXD2-AS1 was detected in stage III-IV HCC patients compared with stage I-II patients (Figure 1C). The associations of FOXD2-AS1 with age, sex, size and stage were summarized in Table 1. It is indicated that FOXD2-AS1 may drive the progression of HCC. To clarify the influence of FOXD2-AS1 on the prognosis in HCC patients, Kaplan-Meier curves were depicted. Worse overall survival was observed in HCC patients expressing high levels of FOXD2-AS1 than those expressing low levels (HR=5.104, $p=0.0239$). As a log-rank test revealed, the overall survival (OS) in high FOXD2-AS1 level group and low FOXD2-AS1 level group was 19.4 and 30.2 months, respectively (Figure 1D). We believed that FOXD2-AS1 was an unfavorable lncRNA to the prognosis in HCC.

FOXD2-AS1 Facilitated Proliferative and Migratory Abilities in HCC

Compared with normal hepatocytes, FOXD2-AS1 was identically upregulated in HCC cells

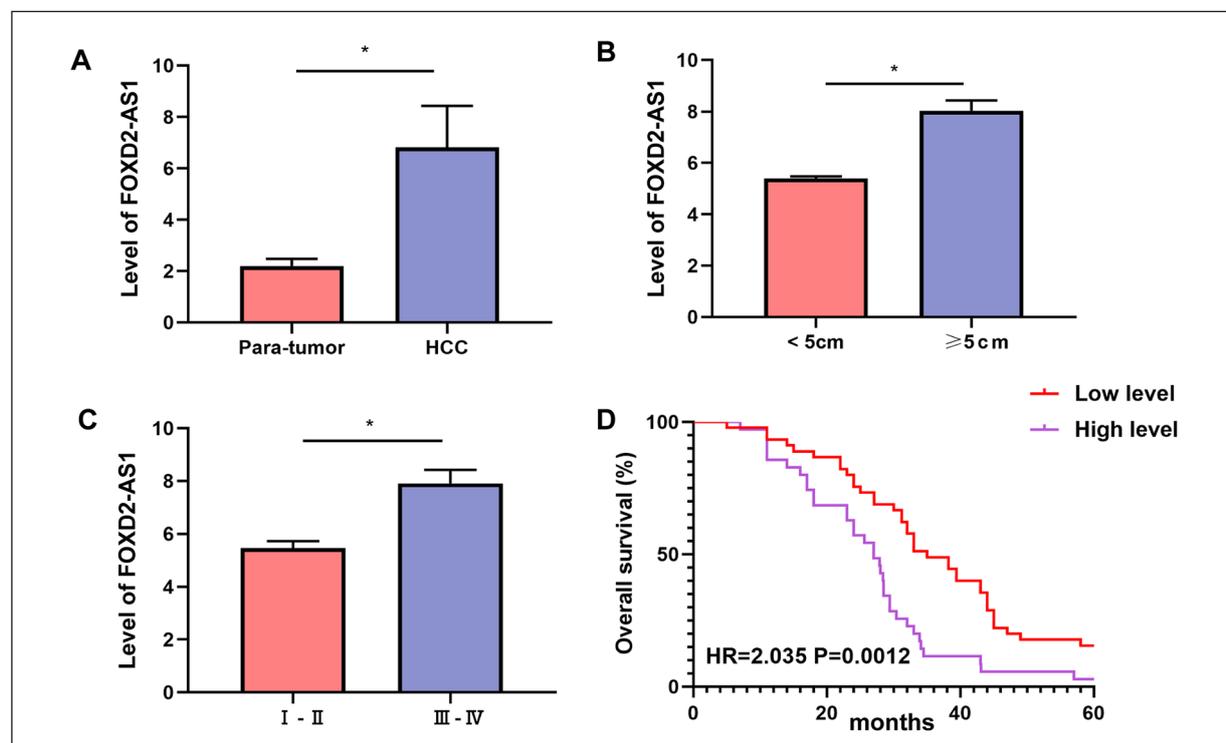


Figure 1. Poor prognosis in HCC patients expressing high levels of FOXD2-AS1. FOXD2-AS1 was upregulated in HCC tissues rather than para-tumor ones. $*p < 0.05$, different from the para-tumor tissues. **B**, FOXD2-AS1 level was higher in larger HCC tissues (≥ 5 cm) than smaller ones (< 5 cm). $*p < 0.05$, different from smaller HCC tissues (< 5 cm). **C**, FOXD2-AS1 level was higher in stage III-IV HCC patients than stage I-II ones. $*p < 0.05$, different from stage I-II ones. **D**, HCC patients expressing high levels of FOXD2-AS1 had poorer prognosis than those expressing low levels (HR=5.104, $p=0.0239$).

Table I. Relationship between the level of FOXD2-AS1, TWIST1 and variable clinicopathological features.

Clinicopathologic features	Number of cases	FOXD2-AS1 expression		TWIST1 expression	
		Low (n = 40)	High (n = 40)	Low (n = 40)	High (n = 40)
Age (years)					
≤ 60	45	21	24	20	25
> 60	35	19	16	20	15
Gender					
Male	38	18	20	20	18
Female	42	22	20	20	22
Tumor size					
< 5 CM	44	29	15	26	18
≥ 5 CM	36	11	25	14	22
TNM stage					
I-II	34	24	10	21	13
III-IV	46	16	30	19	27

HepG2 (Figure 2A). Three FOXD2-AS1 siRNAs were generated, and among them, si-FOXD2-AS1 2# presented the best efficacy to knock down FOXD2-AS1 level in HepG2 cells (Figure 2B). CCK-8 assay showed that the knockdown of FOXD2-AS1

markedly decreased viability in HepG2 cells (Figure 2C). Meanwhile, migratory cell number was reduced after knockdown of FOXD2-AS1 (Figure 2D). Therefore, FOXD2-AS1 was able to stimulate HCC cells to proliferate and migrate.

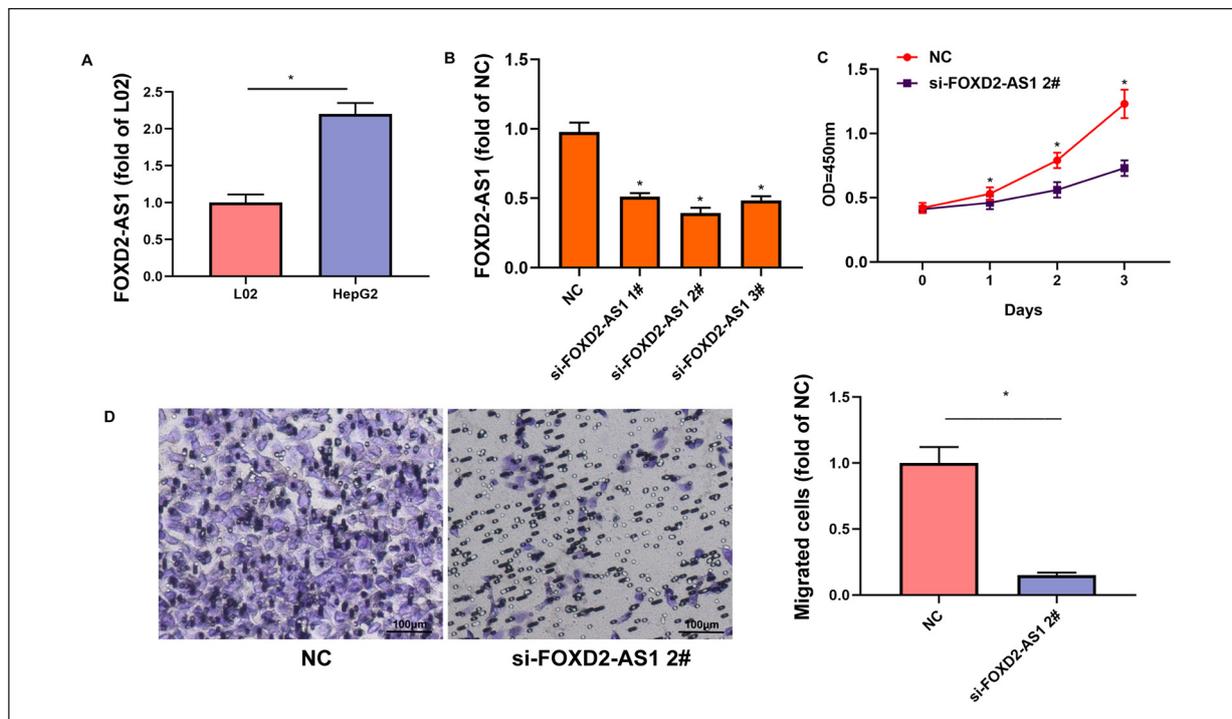


Figure 2. FOXD2-AS1 facilitated proliferative and migratory abilities in HCC. **A**, FOXD2-AS1 was upregulated in HepG2 cells rather than L02 cells. $*p < 0.05$, different from L02 cells. **B**, Transfection of either of three generated FOXD2-AS1 siRNAs effectively downregulated FOXD2-AS1 in HepG2 cells. $*p < 0.05$, different from HepG2 cells treated with NC. **C**, Transfection of si-FOXD2-AS1 2# decreased viability in HepG2 cells. **D**, Transfection of si-FOXD2-AS1 2# inhibited migration in HepG2 cells (magnification: 40 \times) (scale bar = 100 μ m). $*p < 0.05$, different from the HepG2 cells treated with NC.

FOXD2-AS1 Upregulated TWIST1

Subcellular distribution of a lncRNA determines its biological functions. We found that FOXD2-AS1 was mainly expressed in the nucleus (Figure 3A). We also found that TWIST1 was expressed more in the nucleus than in the cytoplasm (Figure 3B). In addition, TWIST1 was upregulated in HCC tissues, indicating the potential involvement of TWIST1 in HCC progression (Figure 3C). The associations of TWIST1 with age, sex, size and stage were summarized in Table I. Pearson correlation test showed a positive correlation between levels of FOXD2-AS1 and TWIST1 in HCC tissues ($r=0.7536$, $p<0.0001$) (Figure 3D). Transfection of si-FOXD2-AS1 2# downregulated TWIST1 in HepG2 cells, further supporting their positive correlation (Figure 3E, 3F).

FOXD2-AS1 Facilitated Proliferative and Migratory Abilities in HCC by Regulating TWIST1

Transfection of pcDNA-TWIST1 markedly upregulated TWIST1 in HepG2 cells, suggesting the well transfection efficacy (Figure 4A).

Interestingly, the inhibited viability and migratory ability in HepG2 cells with FOXD2-AS1 knockdown were partially reversed by overexpression of TWIST1 (Figure 4B, 4C). Collectively, FOXD2-AS1 aggravated the progression of HCC by regulating TWIST1.

Discussion

HCC is the second cause of global cancer death¹⁶. By analyzing HCC profiling using TCGA, Zhao et al¹⁷ found that FOXD2-AS1 is upregulated in HCC tissues and linked to HCC prognosis. Chang et al¹⁸ detected FOXD2-AS1 levels in 140 pairs of HCC and adjacent tissues. They demonstrated that FOXD2-AS1 is upregulated in HCC tissues. In addition, FOXD2-AS1 overexpression triggers *in vitro* proliferative and metastatic abilities in HCC cells. Tumorigenicity assay in nude mice further indicated that highly expressed FOXD2-AS1 triggers *in vivo* growth of HCC. Xu et al¹⁹ also showed that FOXD2-AS1 is upregulated in HCC cells, which is positively correlated to

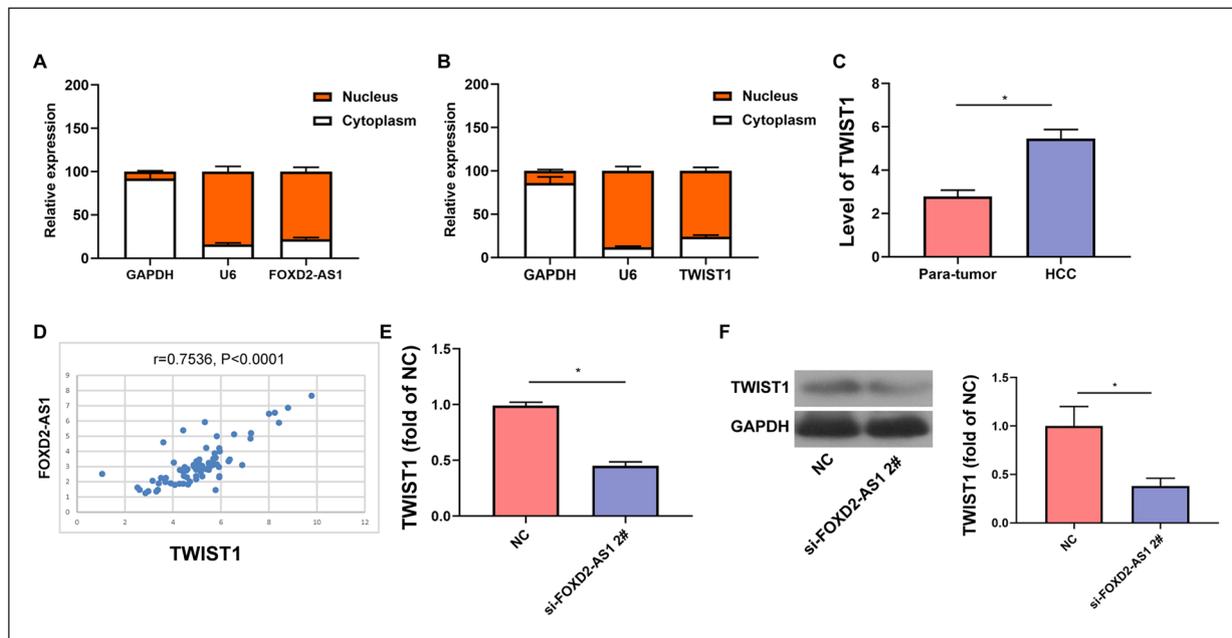


Figure 3. FOXD2-AS1 upregulated TWIST1. **A**, FOXD2-AS1 was mainly expressed in the nucleus. **B**, TWIST1 was mainly expressed in the nucleus. **C**, TWIST1 was upregulated in HCC tissues than para-tumor ones. * $p < 0.05$, different from the para-tumor tissues. **D**, Pearson correlation test showed a positive correlation between expression levels of FOXD2-AS1 and TWIST1 in HCC tissues. **E**, TWIST1 was downregulated in the HepG2 cells transfected with si-FOXD2-AS1 2#. * $p < 0.05$, different from the control cells. **F**, TWIST1 protein expression was downregulated in the HepG2 cells transfected with si-FOXD2-AS1 2#. * $p < 0.05$, different from the control cells.

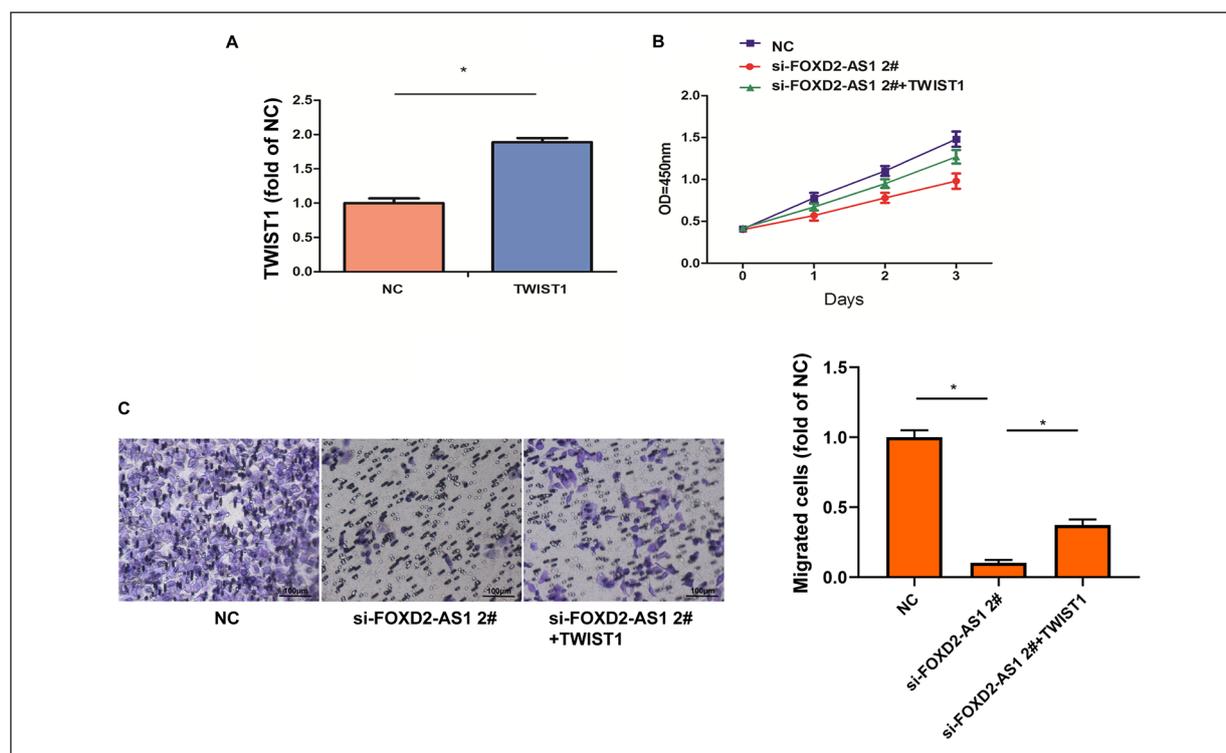


Figure 4. FOXD2-AS1 facilitated proliferative and migratory abilities in HCC by regulating TWIST1. **A**, Transfection of pcDNA-TWIST1 effectively upregulated TWIST1 in HepG2 cells. $*p < 0.05$, different from the HepG2 cells treated with NC. **B**, Overexpression of TWIST1 reversed the inhibited viability in HepG2 cells with FOXD2-AS1 knockdown. $*p < 0.05$, different from the HepG2 cells treated with NC or si-FOXD2-AS1 2#. **C**, Overexpression of TWIST1 reversed the inhibited migration in HepG2 cells with FOXD2-AS1 knockdown (magnification: 40 \times) (scale bar = 100 μ m). $*p < 0.05$, different from the HepG2 cells treated with NC or si-FOXD2-AS1 2#.

tumor number and tumor size. Consistently, our findings uncovered upregulated FOXD2-AS1 in HCC tissues, and its level was linked to the prognosis in HCC patients. Furthermore, *in vitro* experiments demonstrated that FOXD2-AS1 stimulated proliferative and migratory abilities in HCC.

As an important transcription factor, TWIST1 is able to induce EMT in tumor cells^{20,21}. Silence of TWIST1 weakens migration and EMT, thus influencing the progression of colorectal cancer²². In gastric cancer, TWIST1 upregulates FoxM1 by recruiting p300 to the promoter region of FoxM1, thereafter inducing cancer cells to proliferate²³. By targeting the TWIST1/EMT signaling, lncRNA-CHRF stimulates CRC metastasis by downregulating miR-489²¹. Wang et al²⁴ suggested that LUADT1 and TWIST1 are upregulated in NSCLC samples, displaying a positive correlation between each other. Their overexpression drives migratory and invasive rates in NSCLC. In this paper, Pearson correlation analysis proved the positive correla-

tion between expression levels of FOXD2-AS1 and TWIST1 in HCC tissues. Knockdown of FOXD2-AS1 markedly downregulated TWIST1 in HepG2 cells, further supporting their positive interaction. Notably, the overexpression of TWIST1 was able to reverse the inhibited proliferative and migratory abilities in HepG2 cells transfected with si-FOXD2-AS1 2#.

This study for the first time uncovered the molecular mechanism of FOXD2-AS1 in regulating the progression of HCC. FOXD2-AS1 facilitated the progression of HCC by increasing the proliferative and migratory potentials of cancer cells acting on TWIST1 regulation. Our findings may provide some experimental references to HCC pathogenesis and to the development of novel therapeutic targets.

Conclusions

FOXD2-AS1 facilitates the progression of HCC by upregulating TWIST1.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

This study was approved by the Ethics Committee of Guizhou Provincial People's Hospital (No. 2016023).

Informed Consent

Signed written informed consents were obtained from all participants before the study.

Availability of Data and Materials

All data analyzed in this review are included in this article and/or its figures. Further inquiries can be directed to the corresponding author.

Authors' Contributions

All authors contributed to the data collection and contextualization of the paper's contents, critically edited the manuscript, and approved its final version for submission.

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