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ORIGINAL ARTICLE

LncRNA FLVCR1-AS1 accelerates osteosarcoma cells to proliferate, migrate and invade *via* activating wnt/β-catenin pathway

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Summary

Purpose: *To clarify the biological function of long non-coding RNA (lncRNA) FLVCR1-AS1 in the progression of osteosarcoma.*

Methods: *The correlation between FLVCR1-AS1 level and pathological indexes of osteosarcoma patients was analyzed by chi-square test. Subsequently, the regulatory effects of FLVCR1-AS1 on the proliferative, migratory and invasive abilities of osteosarcoma cells were evaluated. Moreover, the relative levels of CTNNB1, SOX4, CCND1, CCND2 and MYC in osteosarcoma cells regulated by FLVCR1-AS1 were detected by qRT-PCR. Finally, rescue experiments were conducted to verify the role of wnt/β-catenin in osteosarcoma progression.*

Results: *LncRNA FLVCR1-AS1 was upregulated in osteosarcoma, which was positively correlated to tumor size, WHO grade and distant metastasis, but negatively correlated to survival of osteosarcoma patients. Overexpression of FLVCR1-AS1 markedly suppressed osteosarcoma cells to proliferate, migrate and invade. Relative levels of CTNNB1, SOX4, CCND1, CCND2, MYC and nucleus β-catenin were upregulated in U2OS and MG63 cells overexpressing FLVCR1-AS1. Finally, CTNNB1 knockdown was identified to reverse the influence of overexpressed FLVCR1-AS1 of osteosarcoma cells.*

Conclusions: *FLVCR1-AS1 accelerates the progression of osteosarcoma via activating wnt/β-catenin pathway.*

Key words: *FLVCR1-AS1, wnt/β-catenin, osteosarcoma*

Introduction

Osteosarcoma is the most common primary bone malignancy in adolescents with high risk of local metastasis and distant metastasis [1]. Blocked differentiation of mesenchymal stem cells or osteoblasts lead to the formation of immature bone structures, manifesting as osteogenic or osteolytic changes with soft tissue masses in the imaging findings [2,3]. Approximately 50% of osteosarcoma patients develop distant metastasis. Clinically, lung metastasis is one of the leading causes of death in osteosarcoma patients [4]. The 5-year survival of osteosarcoma is about 65-70%, and unfortunately, it is only 20% in osteosarcoma patients with lung metastasis [5]. Despite the great effects that have been made on the combination of multidisciplinary treatments, osteosarcoma lung metastasis is still

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a challenge. Therefore, it is urgent to elucidate the molecular mechanisms involved in the invasion and metastasis of osteosarcoma, so as to provide therapeutic strategies for improving the long-term survival.

Long non-coding RNA (lncRNA) is a non-coding RNA with 200 nt long that exerts diverse functions in biological processes [6]. Human Genome Project (HGP) considered that lncRNAs are garbage sequences accumulated during human evolution [7]. However, recent studies have identified the crucial functions of lncRNAs in mediating cellular behaviors [8-10]. LncRNAs have diverse gene regulatory networks for tumorigenesis. Currently, several lncRNAs have been reported to be involved in the progression of osteosarcoma [11]. For example, Zhao et al [12] found that antisense lncRNA HNF1A-AS1 is upregulated in osteosarcoma and promotes cell growth by activating wnt/β-catenin pathway. Sun et al [13] found that lncRNA FGFR3-AS1 promotes the progression of osteosarcoma by activating FGFR3. FLVCR1-AS1 is a novel lncRNA with a positive effect on the development and progression of hepatocellular carcinoma and lung cancer [14,15]. However, its role in osteosarcoma remains unknown.

Dysregulated Wnt pathway has become the focus in tumor biology. It is a complex signaling pathway that promotes tumor development and progression [16]. Abnormal activation of the wnt/β-catenin pathway is associated with tumorigenesis [17]. Regulation of the wnt/β-catenin pathway in osteosarcoma involves multiple secreted protein families, including SFRPs, wnt inhibitory proteins (WIF), DKK, sclerostins, and small molecules [18]. The crucial role of wnt/β-catenin pathway in the progression of osteosarcoma has been well concerned [19]. Ma et al [20] reported that Wnt3a, β-catenin and LEF1 are the main components of wnt/β-catenin pathway, which are involved in the invasion and drug resistance of osteosarcoma. Kansara et al [21] illustrated that in orthotopic osteosarcoma, silenced WIF1 and upregulated β-catenin lead to tumor cell proliferation and dedifferentiation. A recent study found that FLVCR1-AS1 silencing inhibits the proliferative, migratory and invasive capacities of lung cancer cells by suppressing the wnt/β-catenin pathway [21].

Our study mainly explored the role of FLVCR1-AS1 and wnt/β-catenin pathway in the progression of osteosarcoma, which provides a novel therapeutic target for osteosarcoma for clinical application.

Table 1. Sequences of PCR primers

PCR primers	
CTNNB1	Forward:5'-CCAATCTACTAATGCTAATACCTG-3'
	Reverse: 5'-CTGCATTCTGACTTTCAGTAAGG-3'
SOX2	Forward: 5'-CCAAGATGCACAACTCGGAGA-3'
	Reverse: 5'-CCGGTATTTATAATCCGGGTGCT-3'
CCND1	Forward: 5'-TCAGATCTATGGAACACCAGCTCCTGTG-3'
	Reverse: 5'-ATAGATCTTCAGATGTCCACGTCCCGCA-3'
CCND2	Forward: 5'-AGATCTATGGAGCTGCTGTGCCACGA-3'
	Reverse: 5'-AGATCTTCACAGGTCGATATCCCGCAC-3'
MYC	Forward: 5'-CAGCTGCTTAGACGCTGGATT-3'
	Reverse: 5'-GTAGAAATACGGCTGCACCGA-3'
GAPDH	Forward: 5'-AGGTCCACCACTGACACGTT-3'
	Reverse: 5'-GCCTCAAGATCATCAGCAAT-3'

Methods

Sample collection

Osteosarcoma tissues and adjacent normal tissues were harvested from 48 osteosarcoma patients in the First People's Hospital of Tai Zhou. Tissue samples were immediately placed in liquid nitrogen and preserved at -80°C. Clinical data of these patients were collected, including age, gender, tumor size, WHO grade and distant metastasis. The experiment was approved by the Medical Ethics Committee of the First People's Hospital of Lianyungang and signed informed consent was provided by all of the patients.

Cell culture and transfection

Normal osteoblasts (hFOB1.19) and U2OS and MG63 osteosarcoma cell lines provided by Cell Bank, Chinese Academy of Science (Shanghai, China), were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), and preserved in a 37°C, 5% CO_2 incubator.

Cells were seeded in a 6-well plates with 2×10^5 cells/well. Transfection was performed until 70% confluence using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced at 4-6 h.

Cell counting kit-8 (CCK-8) assay

Cells were seeded in 96-well plates with 5×10^3 cells per well. At the appointed time points, fresh medium containing 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was replaced in the wells. After incubation for 2 h, the absorbance at 450 nm was recorded using a microplate reader for plotting the growth curve.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and subjected to reverse transcription. The obtained complementary deoxyribose nucleic acid (cDNA) was amplified using the SYBR Premix Ex Taq (TaKaRa, Dalian, China). The relative level was normalized to that of glyceraldheyde 3-phosphate dehydrogenase (GAPDH). Primer sequences are listed in Table 1.

Transwell assay

Cells were seeded in 24-well plates with 2.5×10^4 cells/well. 200 μL of serum-free medium and 500 μL of complete medium were supplied on the basolateral and apical chamber, respectively. Twelve hours later, Transwell chamber was taken out and the medium was removed. Cells were fixed in 95% ethanol and stained with crystal violet for 20 min. Three randomly selected fields per sample were observed under a microscope for counting the migratory cells (magnification 20×). For the invasion assay, 100 μL of diluted Matrigel was pre-coated in Transwell chamber and 50 μL of fibronectin (FN) (100 μg/mL) was coated at the bottom of the chamber. The other procedures were the same as those of migration assay.

Western blot

Total cellular protein was extracted using radioimmunoprecipitation assay (RIPA) containing phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis. After transferring on a polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland), it was blocked in 5% skim milk for 2 h, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by electrochemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistics

SPSS 20.0 (IBM, Armonk, NY, USA) was used for statistical analyses. Data were expressed as mean ± SD (standard deviation). The t-test was used for analyzing intergroup differences. The survival of osteosarcoma patients was evaluated by x^2 test. The survival curves were plotted according to the Kaplan-Meier method and log-rank test was utilized to search survival differences between the two groups. $P<0.05$ indicated significant difference.

Results

FLVCR1-AS1 was upregulated in osteosarcoma

QRT-PCR data revealed a higher level of FLVCR1-AS1 in osteosarcoma tissues relative to

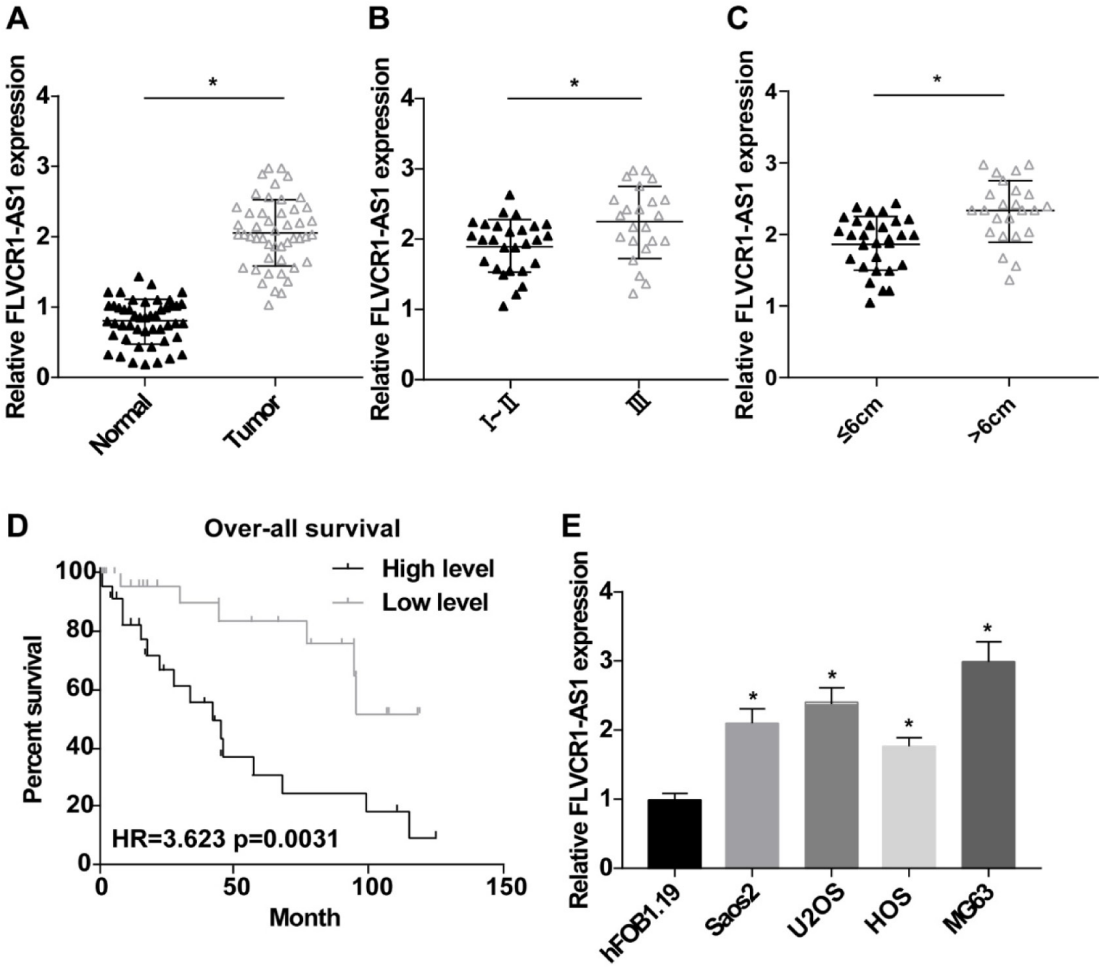

Figure 1. FLVCR1-AS1 was upregulated in osteosarcoma. **A:** Relative level of FLVCR1-AS1 in osteosarcoma tissues and controls examined by qRT-PCR. **B:** Relative level of FLVCR1-AS1 in osteosarcoma tissues in stage III and stage I-II. **C:** Relative level of FLVCR1-AS1 in osteosarcoma tissues ≤ 6 cm and > 6 cm. **D:** Overall survival in osteosarcoma patients with high and low level of FLVCR1-AS1 (HR=3.623, p=0.0031). **E:** Relative level of FLVCR1-AS1 in hFOB1.19 cell line and osteosarcoma cell lines (Saos2, U2OS, HOS and MG63). *p<0.05.

Table 2. Correlation between FLVCR1-AS1 level and pathological characteristics of osteosarcoma patients (n=48)

Clinicopathologic features	*Number of cases*	*FLVCR1-AS1 expression*		*p value*
		Low (n=24)	*High (n=24)*	
Age (years)				0.7726
≤ 20	23	12	11	
> 20	25	12	13	
Gender				0.3861
Male	25	11	14	
Female	23	13	10	
Tumor size, cm				0.0431*
≤ 6	25	16	9	
> 6	23	8	15	
WHO grade				0.0417*
I-II	27	17	10	
III	21	7	14	
Pulmonary metastasis				0.0088*
Yes	21	15	6	
No	27	9	18	

*Asterisks denote statistical significance

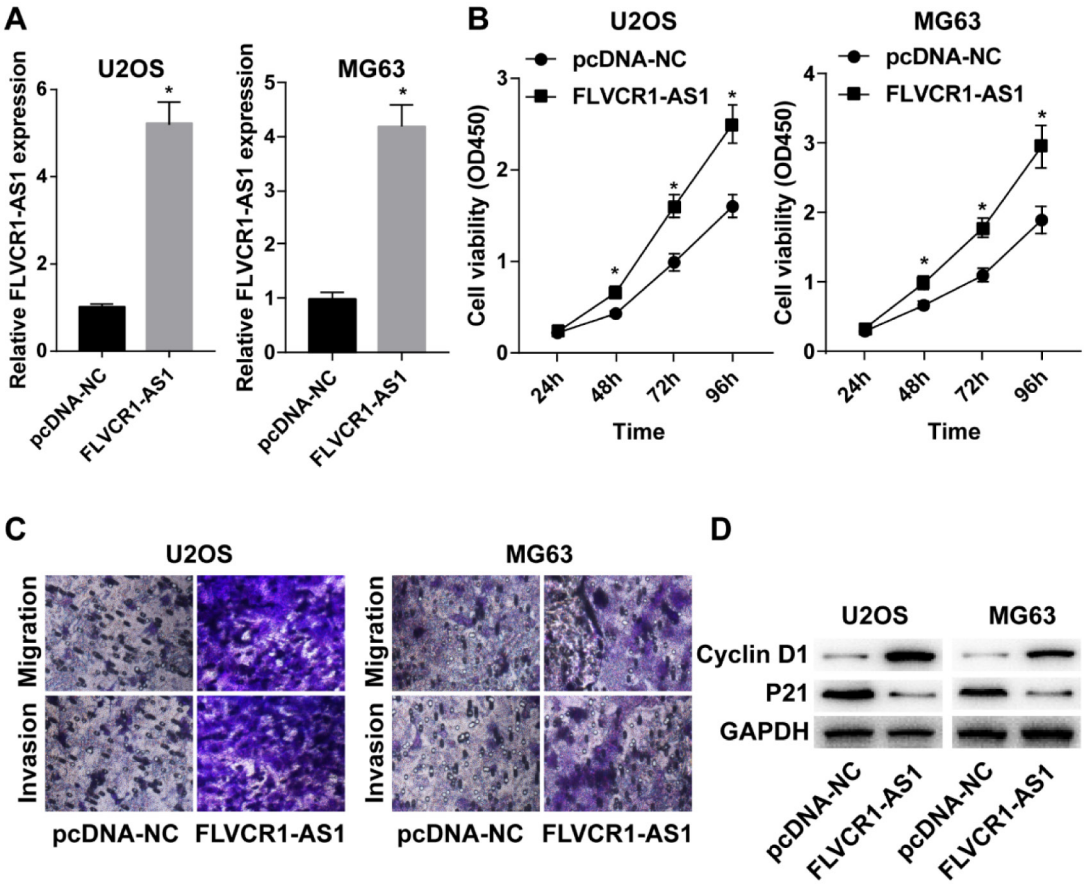

Figure 2. Overexpression of FLVCR1-AS1 accelerated osteosarcoma cells to proliferate, migrate and invade. **A:** Transfection efficacy of pcDNA-NC and pcDNA-FLVCR1-AS1 in U2OS and MG63 cells. **B:** CCK-8 assay revealed the viability in U2OS and MG63 cells transfected with pcDNA-NC or pcDNA-FLVCR1-AS1. **C:** Transwell assay revealed the migration and invasion in U2OS and MG63 cells transfected with pcDNA-NC or pcDNA-FLVCR1-AS1. **D:** Western blot analyses of Cyclin D1 and P21 in U2OS and MG63 cells transfected with pcDNA-NC or pcDNA-FLVCR1-AS1. *p<0.05.

controls (Figure 1A). Furthermore, its level remained higher in stage III osteosarcoma relative to those in stage I-II (Figure 1B). FLVCR1-AS1 showed higher abundance in osteosarcoma tissues larger than 6 cm in size (Figure 1C). X^2 test demonstrated close correlation between FLVCR1-AS1 level with tumor size, WHO grade and distant metastasis of osteosarcoma (Table 2). For analyzing the prognostic data of 48 osteosarcoma patients, those with higher level of FLVCR1-AS1 showed worse overall survival (HR=3.623, p=0.0031, Figure 1D). Subsequently, the cellular level of FLVCR1-AS1 in osteoblasts and osteosarcoma cells was determined as well, which was upregulated in osteosarcoma cell lines (Figure 1E). During the following experiments, U2OS and MG63 cells expressing a relatively high level of FLVCR1-AS1 were selected. The above data demonstrated that upregulated FLVCR1-AS1 may indicate poor prognosis of osteosarcoma.

Overexpression of FLVCR1-AS1 accelerated osteosarcoma cells to proliferate, migrate and invade

Transfection of pcDNA-FLVCR1-AS1 effectively upregulated FLVCR1-AS1 level in U2OS and MG63 cells (Figure 2A). CCK-8 assay revealed the accelerated viability in osteosarcoma cells overexpressing FLVCR1-AS1 (Figure 2B). Besides, Transwell assay identified the increased migratory and invasive capacities after transfection of pcDNA-FLVCR1-AS1 in U2OS and MG63 cells (Figure 2C). Protein level of Cyclin D1 was upregulated, whereas p21 was downregulated after overexpression of FLVCR1-AS1, indicating promoted cell cycle progression (Figure 2D).

Overexpression of FLVCR1-AS1 activated wnt/β-catenin pathway in osteosarcoma

To clarify the molecular mechanism of FLVCR1-AS1 in mediating the progression of osteosarcoma,

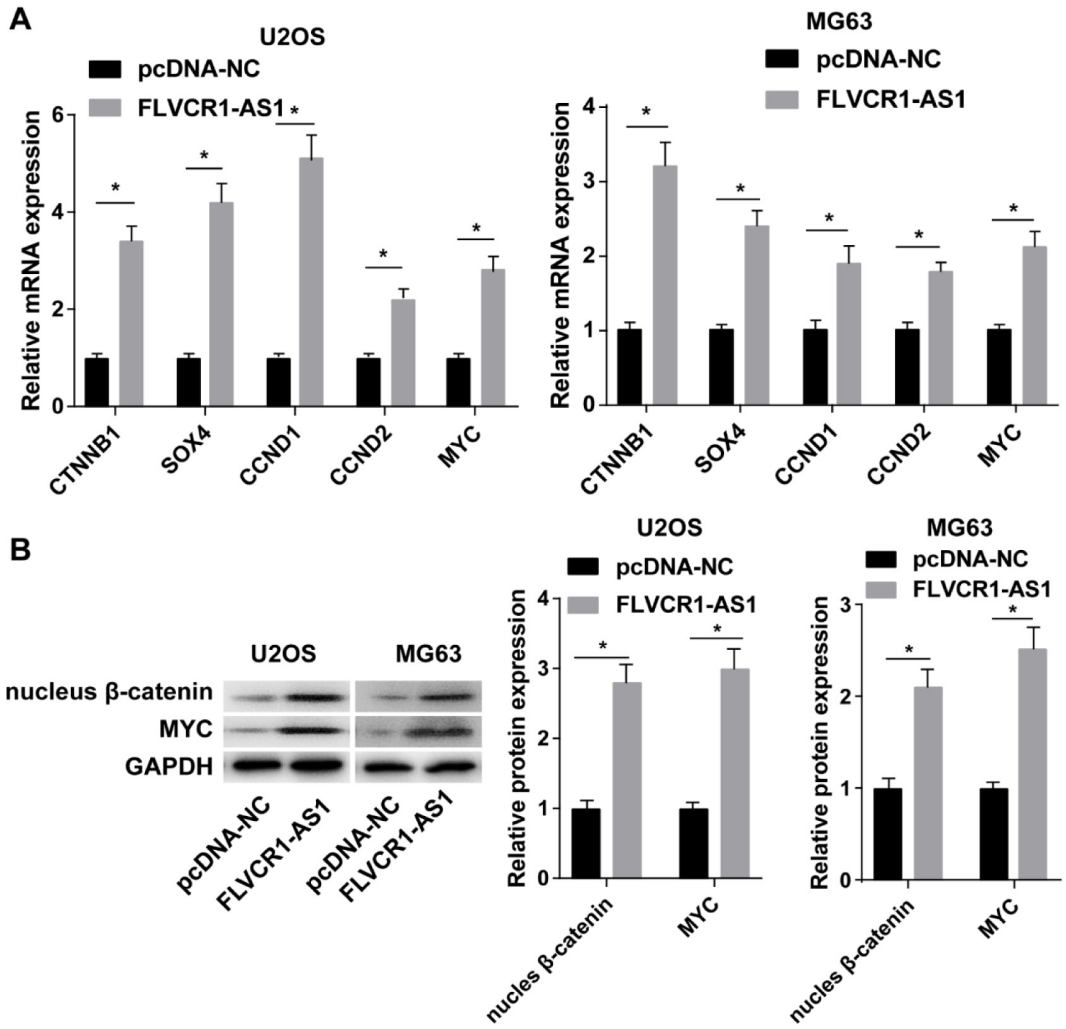

Figure 3. Overexpression of FLVCR1-AS1 activated wnt/β-catenin pathway in osteosarcoma. **A:** Relative levels of CTNNB1, SOX4, CCND1, CCND2 and MYC in U2OS and MG63 cells transfected with pcDNA-NC or pcDNA-FLVCR1-AS1. **B:** Western blot analyses of nucleus β-catenin and MYC in U2OS and MG63 cells transfected with pcDNA-NC or pcDNA-FLVCR1-AS1. *p<0.05.

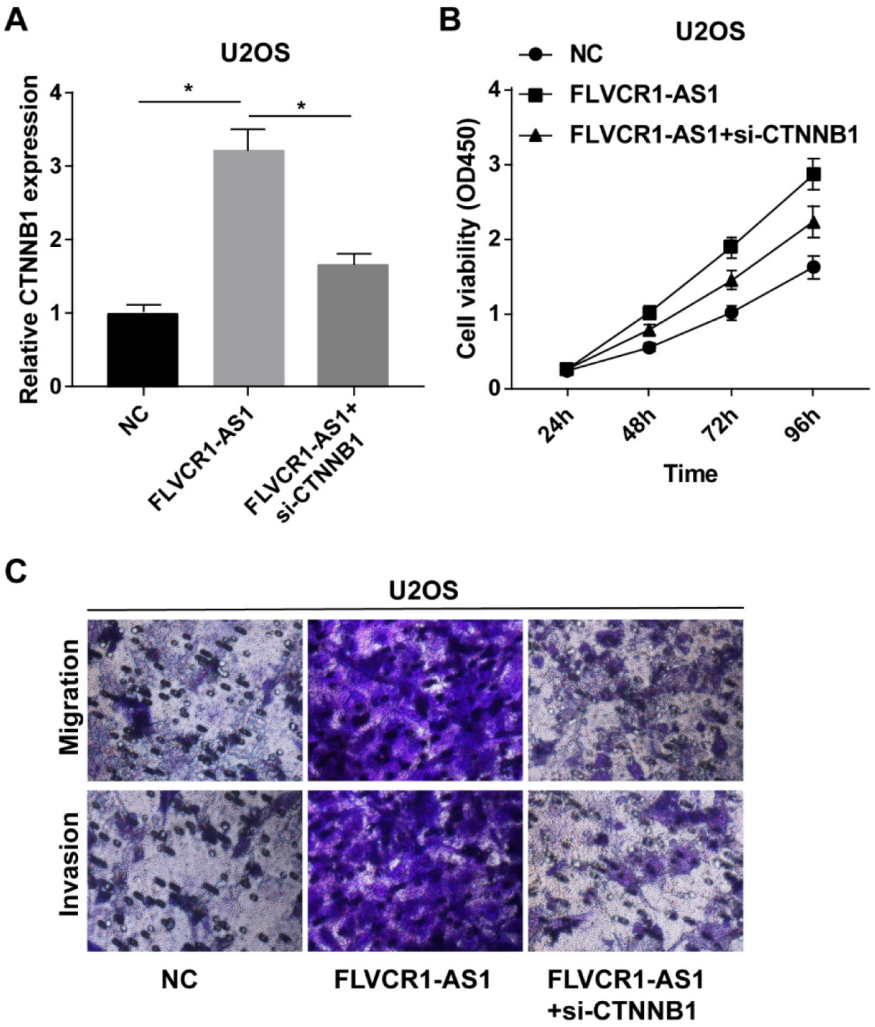

Figure 4. Knockdown of CTNNB1 reversed the biological function of FLVCR1-AS1 in mediating osteosarcoma. U2OS cells were transfected with NC, pcDNA-FLVCR1-AS1 or pcDNA-FLVCR1-AS1+si-CTNNB1, respectively. **A:** Relative level of CTNNB1 in each group. **B:** CCK-8 assay revealed the viability in each group. **C:** Transwell assay revealed the migration and invasion in each group. *p<0.05.

we assessed the expressions of the relative genes in wnt/β-catenin pathway. The mRNA levels of CTNNB1, SOX4, CCND1, CCND2 and MYC were remarkably upregulated after FLVCR1-AS1 overexpression (Figure 3A). Meanwhile, nucleus β-catenin and MYC at protein levels were upregulated in osteosarcoma cells overexpressing FLVCR1-AS1 (Figure 3B), indicating that wnt/β-catenin pathway was activated after overexpression of FLVCR1-AS1.

Knockdown of CTNNB1 reversed the biological function of FLVCR1-AS1 in osteosarcoma

To further verify the role of wnt/β-catenin pathway in FLVCR1-AS1-mediated progression of osteosarcoma, cells were co-transfected with pcDNA-FLVCR1-AS1 and si-CTNNB1. The relative level of CTNNB1 was remarkably reduced after co-transfection of si-CTNNB1 in U2OS cells overexpressing FLVCR1-AS1 (Figure 4A). Notably, the elevated viability due to FLVCR1-AS1 overexpression was partially reduced by knockdown of CTNNB1 (Figure 4B). Identically, the accelerated migratory and invasive capacities of U2OS cells overexpressing FLVCR1-AS1 were reversed by CTNNB1 knockdown (Figure 4C) and it is believed that CTNNB1 knockdown reversed the promotive effect of FLVCR1-AS1 of osteosarcoma cells.

Discussion

Osteosarcoma is common in children and adolescents, with an incidence of 1-3/1000,000 per year worldwide. Osteosarcoma is characterized by high invasiveness and systemic metastasis in the early stage [22]. Lung metastases occur in approximately 10-25% of osteosarcoma patients which it is the leading cause of death from osteosarcoma [23]. However, the prognosis of osteosarcoma has not

improved in the past 30 years due to its genetic and biological complexity. It is of great significance to clarify the pathogenesis of osteosarcoma in order to improve its prognosis.

Recent studies have demonstrated the involvement of lncRNAs in the progression, metastasis and chemotherapy-resistance of tumors acting as oncogenes or tumor-suppressor genes [24]. TUG1 is reported to be a potential therapeutic target for osteosarcoma [25]. Upregulation of UCA1 is closely related to tumor enlargement and distant metastasis, serving as a hallmark for predicting poor prognosis [26]. Zhang et al [14] revealed that FLVCR1-AS1 is upregulated in liver cancer, and positively correlated to the disease severity. Knockdown of FLVCR1-AS1 would impair the proliferative, migratory and invasive capacities of liver cancer cells. Gao et al [15] proposed that FLVCR1-AS1 could be utilized as novel biological marker and therapeutic target for NSCLC. In this study, FLVCR1-AS1 was highly expressed in osteosarcoma, which accelerated osteosarcoma cells to proliferate, migrate and invade. In addition, FLVCR1-AS1 overexpression upregulated Cyclin D1 and downregulated p21 in U2OS and MG63 cells.

Subsequently, we identified that FLVCR1-AS1 overexpression upregulated key genes in wnt/β-catenin pathway, as well as nucleus β-catenin and MYC. Activated wnt pathway is closely related to tumorigenesis [27]. As the key molecule in wnt pathway, dysregulated β-catenin participates in the occurrence and progression of osteosarcoma [28,29]. β-catenin locates on 3p21-22 gene and is encoded by CTNNB1. Functionally, β-catenin is capable of mediating intracellular adhesion, differentiation, embryonic development and tumorigenesis [30]. Wnt/β-catenin can coordinate in the proliferation and differentiation of osteoblasts, which are important behaviors in triggering tumorigenesis of osteosarcoma [31]. The relative level of β-catenin is closely related to distant tumors' metastasis. In the classical wnt/β-catenin pathway, the binding of wnt to membrane receptors frizzled and LRP5/6 stimulated the cytoplasmic accumulation of β-catenin, and further induces the nuclear translocation of β-catenin to activate downstream oncogenes *via* TCF/LEF pathway [32]. Subsequently the activated oncogenes, such as C-MYC, MYB, CCND and CCNE, accelerate tumor cell growth [33]. Our results also indicated that knockdown of CTNNB1 could reverse the effect of overexpressed FLVCR1-AS1 on osteosarcoma cells.

Conclusions

To sum up, this study illustrated that overexpression of FLVCR1-AS1 accelerated osteosarcoma cells to proliferate, migrate and invade through activating wnt/β-catenin pathway, thus promoting the progression of osteosarcoma.

Conflict of interests

The authors declare no conflict of interests.

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