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Original Article

FH535 suppresses the growth of osteosarcoma in vitro and vivo via downregulation Wnt/β-catenin signaling pathway

Yao Lu[1*], Na Nie[2*], Chunqiu Wang[3*], Qian Wang[1], Qiang Li[1], Teng Ma[1], Bin Hu[4], Yong Zhou[4], Zhong Li[1], Dingjun Hao[1], Kun Zhang[1]

[1]Department of Orthopaedic Surgery, Hong Hui Hospital, Xi'an Jiaotong University College of Medicine, Xi'an 710054, China; [2]Department of Nephrology, The Center Hospital of Han Zhong, Hanzhong 723000, China; [3]Department of Orthopaedic Surgery, Chinese People's Liberation Army 359th Hospital, Zhenjiang 212001, China; [4]Department of Orthopaedic Surgery, Tangdu Hospital, The Fourth Military Medical University, Xi'an 710038, China. [*]Equal contributors.

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Abstract: Osteosarcoma (OS) is a rare malignancy in the pediatric age group leading causes of cancer-related death. In recent years, increasing studies evaluated the inhibition of the Wnt/β-catenin pathway as a potential therapeutic approach. Previous studies reported that Wnt/β-catenin pathway inhibitor FH535 has been shown to suppress tumor progression in various tumors, including lung, prostate and breast cancer. However, there are few studies illustrating the inhibitory effect of FH535 on OS. To test this, we first demonstrated that FH535 inhibits the OS cells (F5M2) proliferation using MTT assay. We then found FH535 decreased invasion and migration ability of F5M2 through transwell assays, and the expression of cyclin D1 and MMP-2 were both down-regulated in FH535 group compared with the control group by western blot. In addition, FH535 suppressed OS growth, reducing the size of OS F5M2 cell xenografts. Together, these results demonstrated that FH535 suppresses the tumor growth of OS in vitro and vivo, suggesting that FH535 may be a promising therapeutic agent for the treatment of OS.

Keywords: Osteosarcoma, FH535, Wnt/β-catenin, growth

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in children and adolescents. This tumor is generally locally aggressive and tends to produce early systemic metastases [1]. Chemotherapy in conjunction with surgery achieves a 5-year event-free survival of 60-70% in extremity localized, non-metastatic disease, While a total of 30-40% of patients with localized OS will develop a local or distant recurrence [2]. Five-year overall survival (OAS) for recurrent OS has been reported to be 23-29% [2]. Surgery is the treatment of choice for relapsed patients whenever possible, but addition of chemotherapy to surgery provides survival benefit in patients not achieving second surgical remission [3]. Cancer cell invasion and metastasis lead to a poor prognosis and are a therapeutic challenge [4]. Therefore, the identification of the critical molecules and/or signal transduction pathways responsible for suppressing development of invasion/metastasis is significant for the development of novel treatment strategies for this kind of cancer.

β-catenin is a key component protein of the Wnt/β-catenin signaling pathway which is one of the fundamental pathways directing cell proliferation, cell polarity and cell fate determination during embryonic development and tissue homeostasis [5]. Remarkably, β-catenin is aberrantly activated in more than 70% of colorectal cancers [6] and to a lesser extent in other tumor types, such as pancreatic, breast and gastric cancer [6-9]. In the nucleus, β-catenin binding to the T cell factor/lymphoid enbancer factor (TCF/LEF) family of transcription factors serves as an activator leading to an increased expression of downstream target

genes, including cyclin D1, c-myc, MMPs which regulate cell cycle, growth, and progression [10, 11]. Meanwhile upregulation MMPs increases the degradation of extracellular matrix (ECM) and promote the invasion and metastasis of tumor cells [12]. Accordingly, β-catenin has gained recognition as an enticing molecular target for cancer therapeutics. Disruption of protein-protein interactions essential for β-catenin activity holds immense promise for the development of novel method to anti-cancer.

It is reported that β-catenin protein expression was greater in osteosarcoma compared with osteochondroma, and its expression level positively correlated with an increasing order of tumor malignancy (clinical Ennecking stage, pathological degree, pulmonary metastasis) [13]. The results suggested that β-catenin enhances tumor progression and metastasis in OS cells. Therefore, target of the Wnt/β-catenin pathway by small molecules could be a promising therapeutic approach for cancer cells in which this pathway is activated. In order to verify this hypothesis, we adopted FH535 an inhibitor of the β-catenin pathway [14] as a tool to evaluate its ability to inhibit growth, migration, and invasion of OS cell lines (F5M2), and suppressed the tumor growth of OS in vivo. Additionally, the study aimed to assess matrix metalloprotease (MMP) and Wnt/β-catenin signaling pathways as potential mechanisms for FH535 regulation OS progression.

Materials and methods

Cell culture and FH535 treatment

Human OS cell subline F5M2 was established and maintained in our laboratory as previously described [15] and grown in RPMI-1640 (HyClone, Thermo Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 μg/mL), and glutamine (2 mM). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. FH535 was purchased from RD, and dissolved in DMSO (Sigma) at a concentration of 10 mM as a stock solution and used bydiluting in RPMI-1640 to give a final concentration [16].

Cell proliferation assay

The effect of FH535 on the proliferation of OS cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT (Promega) assay. A total of 3000 cells/well in 0.2 mL complete medium were seeded in a 96-well plate and treated with FH535 at a concentration ranging from 0 to 100 μM for 24 h, 48 h and 72 h as previously described [17]. After maintained at 0.1 mL medium at 37°C for 60 min and 20 μl MTT for 4 h, add 150 μl DMSO. Then measure Optical density (OD) at 490 nm on a microplate reader. All treatment concentrations were repeated in three wells.

Transwell cell migration and matrigel invasion assays

Tumor cell invasion was characterized using the Transwell systems (Coning). Briefly, the cells were harvested and resuspended in serum-free RPMI 1640 medium with various concentrations of FH535 (0, 30, 60, and 100 μM) at a concentration of 1×10^5 cells/ml. Then add 100 μL migration medium into the upper chamber of Transwell system which was coated with 50 μL diluted matrigel and maintained at 37°C for 4 h. The lower chambers were filled with 500 μL RPMI-1640 medium containing 10% fetal bovine serum. After the cells were incubated for48 h, the non-invading cells that left at the upper surface were removed with a cotton swab. All the other procedures were performed as previously described [18]. The experiments were performed in triplicate and repeated three times independently.

The procedure of transwell migration assays were the same as the transwell invasion assays except that the upper chamber was not coated with matrigel.

Western blot

F5M2 cells (7×10^5) were cultured in RPMI-1640 + 10% FBS in 6 well plates for 72 h. After renew fresh medium, the cells were treated with 0, 30, 60, and 100 μM of FH535 for 38 h [19]. Protein concentrations in the cell extracts were assayed with BCA protein assay kit according to provider's protocol (Thermo Scientific Pierce). Polyclonal antibody against β-catenin was bought from Cell Signaling Technology (1:1000, Beverly, Massachusetts, USA). Polyclonal antibody against cyclin D1, MMP2 were purchased from Abcam (Cambridge, UK). Peroxidase-conjugated goat anti-rabbit IgG and monoclonal against β-actin were purchased

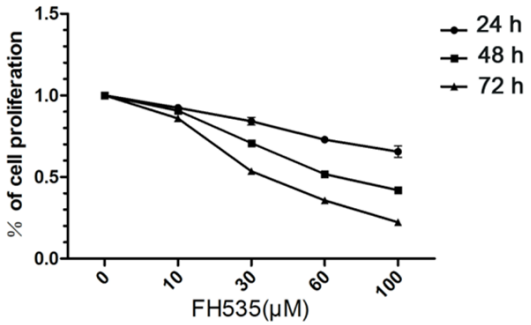

Figure 1. FH535 inhibits OS cell proliferation in concentration-dependent and time-dependent manners. Incubation of F5M2 cells with FH535 for 24 h, 48 h, 72 h inhibits proliferation of cells in a concentration-dependent manner compared tonon-FH535-treated control cells (P<0.05). Assay was repeated three times and representative pictures are shown.

from Bioworld Technology (Minnesota, USA). All the other procedures were performed as previously described [17].

Animal studies

This study was approved by the Animal Ethics Committee of Hong Hui Hospital, Xi'an Jiaotong University College of Medicine. Four-week-old female BALB/c nude mice were purchased from the Experimental Animal Centre of the Fourth Military Medical University (Xi'an, China), and maintained under specific pathogen-free condition.

Orthotopic tumors were established by injecting 1×10^6 F5M2 cells in 100 μl into the proximal tibia of each anesthetized nude mouse. When orthotopic tumors reached a size of 100 mm^3, the xenografted nude mouse were randomly divided into control (vehicle) and FH535 (20 mg/kg) groups. FH535 was intraperitoneally administered three per week for 6 weeks. The lendth, width and height of individual orthotopic tumors were measured every 7 days with calipers, and the volume (mm^3) was calculated according to the formula: π/6 × length × width × height. The mouse was sacrificed at 42 day after inoculation, and orthotopic tumors were harvested and weighed. cyclinD expression levels in the tumors were tested using real-time quantification PCR (RTq-PCR).

Statistical analysis

Statistical analyses were performed using the SPSS 19.0 software (SPSS, Inc.) and represented as mean ± standard deviation. In each case P<0.05 was considered statistically significant. Statistical analysis was performed using one-way analysis of variance or two-tailed Student's t-test for comparison between groups. In each case P<0.05 was considered statistically significant.

Result

FH535 inhibits proliferation of F5M2

Numerous studies has demonstrated that the compound FH535 was toxic to a number of cell lines and inhibited cell proliferation, including Huh7, HCT-116, HepG2 and so on [14]. To confirm whether OS cell lines was affected by this compound, F5M2 were cultured with various concentration of FH535 (0, 30, 60, 100 μM) for 24 h, 48 h and 72 h. As shown in **Figure 1**, relative to untreated cells (non-FH535), treatment of cells reduced the proliferation capacity in a dose-dependent manner after the treatment of cells for 24 h (P<0.05). Meanwhile, proliferation decreased with increasing amounts of time. This data indicates that FH535 does indeed inhibit cell proliferation in dose-dependent and time-dependent manners.

FH535 decreased invasion and migration of F5M2

Invasive growth is an important biological characteristic of malignant tumor cells. To determine the impact of FH535 worked on F5M2 cell invasion, an in vitro Matrigel invasion assay was employed. Following treatment with different concentration of FH535 (0, 30, 60, 100 μM) for 48 h, a general reduction in cell invasion was observed as shown in **Figure 2** (P<0.05). The cell line appeared to significantly impact on the invasiveness of the cells in a dose-dependent manner. The result of transwell migration assays was the same as the transwell invasion assays.

Expression of β-catenin target genes cyclin D1 and MMP-2 was inhibited by FH535

In order to verify mechanisms of tumor growth, migration and invasion which were inhibited by FH535 through β-catenin signaling pathway, we focused on its down-stream targets genes cyclin D1 and MMP-2 which were commonly

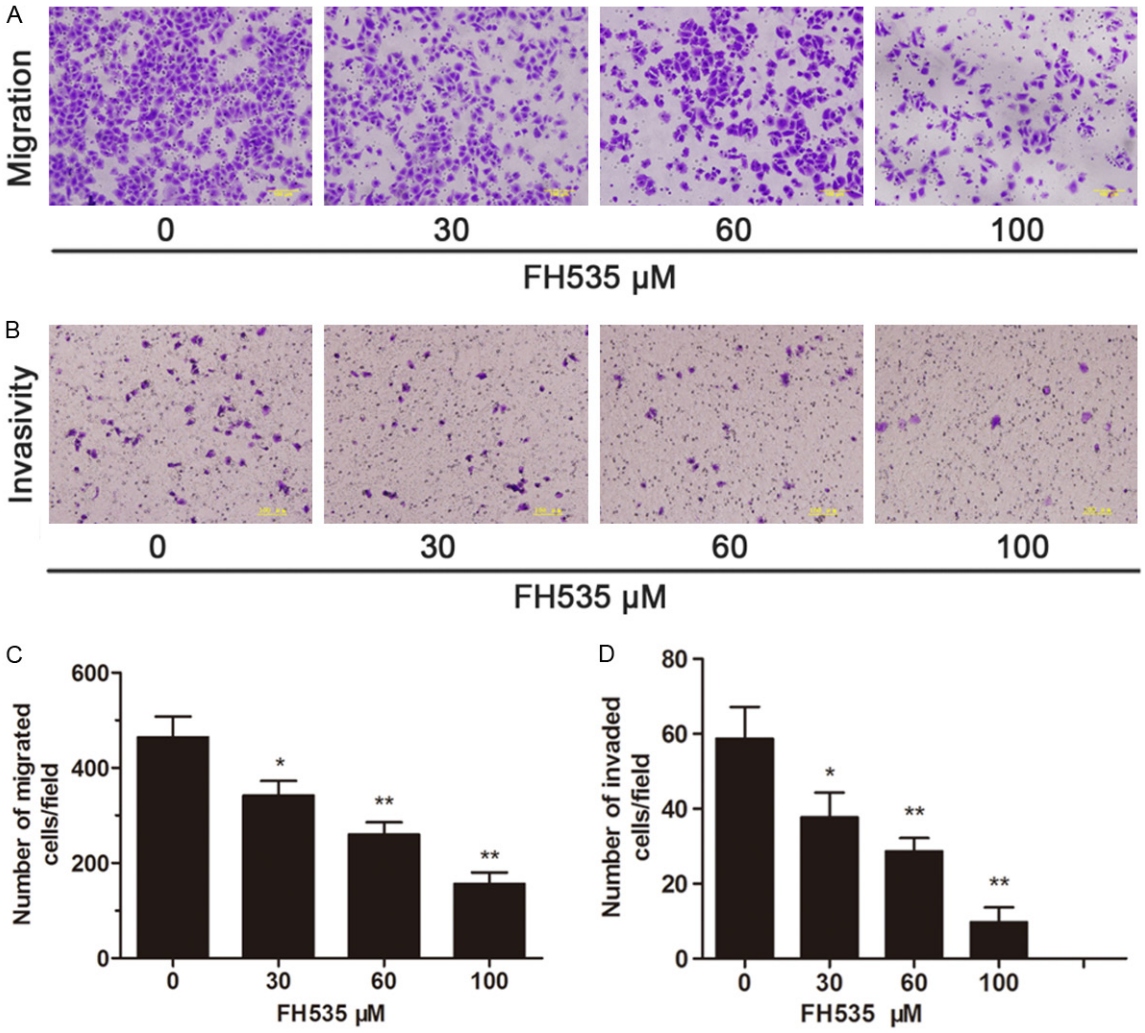

Figure 2. FH535 treatment decreases the invasion and migration in F5M2 cell line. F5M2 were cultured with various concentration of FH535 (0, 30, 60, 100 μM) for 48 h and was determined using Transwell assays. A, C. Treatment with FH535 resulted in reductions in the migration potential at a concentration-dependent manner in comparison to non-FH535-treated cells. B, D. Treatment with FH535 resulted in reductions in the invasion potential at a concentration-dependent manner in comparison to non-FH535-treated cells. The migratory cells were counted under microscope and the results are summarized and expressed as the mean number of migratory cells ± SD per microscopic field. Significant inhibition versus control, *P<0.01, **P<0.001. Migration assays were repeated three times and representative pictures of cell migration are shown.

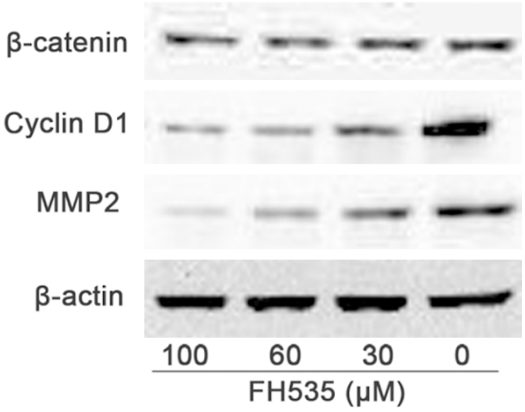

Figure 3. Effect of FH535 on β-catenin and its signaling molecules in F5M2 cells. F5M2 cells were cultured in the presence or absence of FH535 (0, 30, 60, 100 μM) for 38 hat 37°C. Western analysis revealed that both Cyclin D1 and MMP-2 protein levels were reduced in a dose-dependent manner compared to control cells which were not treated with FH535, but the expression of β-catenin is not changed.

associated with cancer invasion, migration and metastasis to account for reduced cell invasion and motility. Western analysis revealed that treatment of F5M2 cells with FH535 for 38 h,

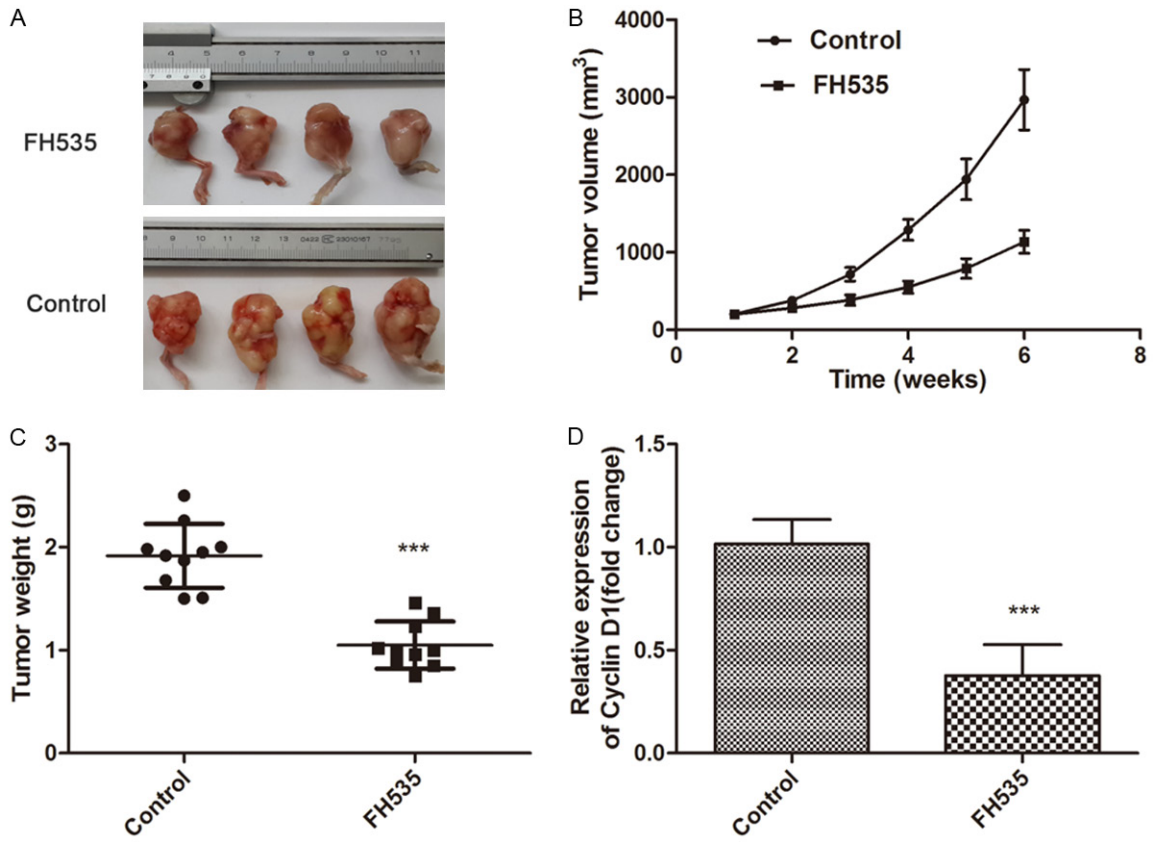

Figure 4. FH535 inhibits tumor growth of OS in vivo. A. Representative photographs of tumors from the untreated (control) and YM155 treatment group. B. Tumor growth curves measured after the inoculation. C. Tumor weight from control and YM155 treatment group, ***P<0.001. D. Forty-two days after inoculation, Cyclin D1 levels were determined in orthotopic tumors by RTq-PCR. Columns, mean of three independent experiments; bars, SD; ***P<0.001.

both Cyclin D1 and MMP-2 protein levels were reduced in a dose-dependent manner compared to control cells which were not treated with FH535, as shown in **Figure 3**. Interestingly, the expression of β-catenin is not changed.

FH535 inhibits tumor growth of osteosarcoma in vivo

The mice were sacrificed 42 days after post-inoculation, and orthotopic tumor tissue was excised. Treatment with FH535 obviously abolished tumor growth (**Figure 4A** and **4B**). FH535 treatment markedly reduced orthotopic tumor weight compared with the control (P<0.05). The mean tumor weight at study termination was 1.05±0.07 and 1.92±0.10 g in the YM155 and control group (**Figure 4C**). Orthotopic tumors in the FH535 group expressed lower cyclin D1 levels compared with control group (**Figure 4D**) indicating that FH535 significantly inhibited the tumor growth in vivo.

Discussion

Primary OS is the most common bone tumor occurring in childhood and adolescence. It has been reported to be the third most common cancer in adolescence, occurring less frequently than only lymphomas and brain tumors in this age group [20]. An unknown etiology, high genetic instability of OS cells, a wide histological heterogeneity, lack of biomarkers, high local aggressiveness, and a rapid metastasizing potential create it difficult to obtain meaningful progress in patient survival [2]. Although great progress has been made during the last two decades by combination chemotherapy and aggressive surgical resection in the treatment of OS, the survival rate of OS patients with localized disease at diagnosis has plateaued at ~70%, and the long-term survival rate of patients with metastatic or recurrent disease remains at <20% [21]. To a certain extent, the reason behind this may be ascribed to the che-

moresistance to anti-OS therapy. Chemoresistance in OS appears to be mediated by numerous mechanisms, which include decreased intracellular drug accumulation, drug inactivation, enhanced DNA repair, perturbations in signal transduction pathways, apoptosis-and autophagy-related chemoresistance, and so on [22]. At present, the majority of the studies focus on perturbations the critical molecules and/or signal transduction pathways responsible for regulating the proliferation and growth of OS cells, such as IGF-1R antibodies, VEGFR, PDGFR, Ras/ERK/(MAPK) signal pathway, mTOR signal transduction pathways [23, 24], leading to the development of a variety of novel targeted therapeutic agents for OS. Hence, identification the targeted molecules of signal transduction pathways that regulate OS cell proliferation and growth and the interaction between these pathways will lead to the development of numerous novel targeted agents.

β-catenin encoded by CTNNB1 gene is part of a complex of proteins has two main functions: i) It is a structural adaptor protein that links cadherin to the actin cytoskeleton, thus, it plays an important role in cell-cell adhesion; ii) It is also a pivotal signaling molecule acting downstream of the Wnt signaling cascade [10, 25]. While, the wnt/β-catenin signaling pathway is a significant pathway that regulates cell proliferation, migration, and differentiation, thus making it a powerful regulator of embryonic development and tumorigenesis [26]. In the absence of Wnt, β-catenin forms a complex with GSK-3b, Axin and APC and is phosphorylated by mainly GSK-3b. Phosphorylated β-catenin is conjugated with ubiquitin and then degraded by proteosome [27]. In the presence of Wnt ligand, β-catenin is not ubiquitinated and accumulates in cytoplasm. Accumulated β-catenin translocates into the nucleus and then binds to the TCF/LEF family of transcription factors to coactivate target genes [27]. This translocation results in the expression of downstream oncogenic genes, such as cyclin D1, c-myc, MMPs [10], involved in the regulation of cell adhesion, proliferation, invasion. Interestingly, numerous studies reported that up to 70% of colorectal cancers and to a lesser extent in other tumor types, such as colon cancer, breast and gastric cancer [6-9], observed β-catenin aberrantly activated, suggesting that protein β-Catenin is thought to play a crucial pathogenetic role. Therefore, targeted β-catenin may be an effective therapeutic approach for OS. In order to verify this hypothesis, we adopted FH535 an inhibitor of the β-catenin pathway as a tool to evaluate its ability to inhibit growth, migration, and invasion of OS cell lines (F5M2).

FH535, a small molecule inhibitor of the β-catenin/TCF4 complex, has been shown to suppress nuclear translocation of β-catenin [28]. Consequently, reduced nuclear β-catenin expression was due to the inhibition of the target genes transcriptional activity. When FH535 effects on cell, β-Catenin protein expression in cells is not affected while the transcription complex TCF/LEF binding activity was significantly suppressed [14]. This study used FH535 intervention Wnt/β-catenin signaling pathway activation. Based on our observation, relative to untreated cells (non-FH535), treatment of groups reduced the proliferation, invasion and migration capacities of the OS cell line F5M2 in dose-dependent manners, whereas the level of β-catenin protein expression was slightly changed after the treatment by FH535, consistent with the previous studies [15, 29].

Cyclin D1 is a proto-oncogene that plays a role in cell proliferation through activation of cyclin-dependent kinases CDK4 and CDK6 [30], which phosphorylate the retinoblastoma protein and thus advance through the G1 phase of the cell cycle, leading to stimulation of DNA synthesis [31]. Dysregulation of cyclin D1 gene expression or function contributes to the loss of normal cell cycle control leading to tumorigenesis. Previous study reported that amplification or overexpression of cyclin D1 play spivotal roles in the development of a subset of human cancers including parathyroid adenoma, breast cancer, colon cancer, lymphoma, melanoma, and prostate cancer [32]. Roberto Gedaly finds that Wnt/β-Catenin signaling pathway promoting the cell proliferation may be associated with transcriptional activation of cyclin D1 [19]. In agreement with these results, in the present study, our results showed that FH535, a small-molecule inhibitor of Wnt/β-catenin signaling pathway, significantly suppressed OS growth in vitro and in vivo, suggesting that FH535 suppress β-catenin transcription activity resulting in downregulation of wnt/β-catenin signaling pathway target gene cyclin D1 transcription and inhibition the OS cell proliferation.

Tumor metastasis is a complex multi-step process and a critical event in tumor cell invasion is the degradation of the extracellular matrix (ECM) and basement membrane that act as a barrier to the spread of cancer cells to distal sites by restricting tumor growth and invasion. MMPs are endopeptidases and the classical function is the degradation of ECM [33]. Degradation of ECM components by MMPs facilitates invasive growth and metastasis of tumor cells, via blood and lymphatic routes around the body [33]. Especially MMP-2 (gelatinase A) and MMP-9 (gelatinase B) which play a key role in degradation of type IV collagen and gelatin, the two main components of ECM, play crucial roles in most tumor invasion and metastasis [34, 35]. Frequently activated pathways in cancer such as RTKs, Wnt/β-Catenin and TNF-α can upregulate MMPs expression at them RNA and protein levels [33, 36, 37]. As a possible mechanism to account for the enhanced cell invasion and motility seen in β-catenin-targeted OS cells, the expression level of MMP-2 was examined in our current study. Based on our observation, compared to untreated group (non-FH535), the expression level of MMP-2 proteins which were treated with FH535 reduced in a dose-dependent manner. This finding showed that blocking β-Catenin activity in F5M2 cells resulted in the suppression of MMP-2 expression, demonstrating that FH535 are direct targets of β-catenin involved in β-catenin-dependent invasive growth and metastasis in OS cells.

In summary, expression and function of the β-catenin signaling target cyclin D1 and MMP-2 were down-regulated by FH535. The outcome of this study suggests that FH535 has the ability to block or inhibit the proliferation, invasion and migration ability of potential of OS cells in vitro through inhibition of cyclin D1 and MMP-2, as well as suppresses tumor growth in vivo through inhibition of cyclin D1. FH535 inhibits the Wnt/β-catenin signaling pathway, which contributes to inhibition of OS cell survival. These findings suggest that FH535 may serve as the basis for chemoprevention or therapy of malignant OS in high risk individuals.

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Disclosure of conflict of interest

None.

Address correspondence to: Kun Zhang, Dingjun Hao and Zhong Li, Department of Orthopaedic Surgery, Hong Hui Hospital, Xi'an Jiaotong University College of Medicine, The East Road of You Yi, Xi'an 710054, China. Tel: +8615609213078; E-mail: gukezk@163.com (KZ); Tel: +8615308343056; E-mail: gukehdj@sohu.com (DJH); Tel: +8613992-888878; E-mail: lizhong0607@126.com (ZL)

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