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Original Article

Crosstalk between Hh and Wnt signaling promotes osteosarcoma progression

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Abstract: Objective: Osteosarcoma (OS) is the most common primary malignant tumor of bone. Patients with localized osteosarcoma are routinely treated with chemotherapy and surgery. However, many patients eventually relapse after these treatments. Therefore, it is important and urgent to identify better therapeutic strategies. Hedgehog-GLI is responsible for the development of bone and tumorigenesis. Aberrant activation of GLI-2 is correlated with various malignancies including OS. Methods: RT-QPCR and western blot were performed to detect the expression of GLI-2 among human osteosarcoma cell lines U2OS, SaOS and human osteoblast cells HOB-c. siRNA or overexpression method were used to knock down/overexpress GLI-2 or β-catenin and MTS formazan generation method were applied to study the function of GLI-2 and β-catenin in the proliferation of OS cells. Results: We showed that GLI-2 is highly expressed in osteosarcoma cell lines. Knockdown of GLI-2 by siRNA decreases osteosarcoma cell proliferation. Further, we showed that knockdown of GLI-2 can decrease the protein level of β-catenin, and β-catenin depletion by siRNA could decrease the proliferation of OS cells. Interestingly, overexpression of β-catenin in GLI-2 knockdown cells partially increased proliferation. Conclusion: These findings suggest Hh/GLI-2-Wnt/β-catenin crosstalk is required for osteosarcoma cell proliferation. GLI-2 may play a role in the regulation of the expression of β-catenin in OS cells and β-catenin may function as downstream effector of crosstalk between Hh and Wnt signaling. GLI-2 may be exploited as a therapeutic target for the treatment of osteosarcoma patients.

Keywords: Wnt, Hedgehog, osteosarcoma, β-catenin, GLI-2

Introduction

Osteosarcoma (OS) is one of the most common malignant bone tumors in childhood and adolescence and the second leading cause of mortality in this age group [1, 2]. Most deaths occur due to recurrence or metastasis to distant organs even treated maximally with chemotherapy [3, 4]. Obviously, to improve clinical outcomes and cure OS, we need better understanding of the mechanisms underlying its pathogenesis, progression, invasiveness, relapse, metastasis and chemo-resistance.

Hedgehog (Hh) signaling pathway has been widely shown important in several stages of vertebrate embryonic development as well as in tumorigenesis of multiple cancers [5-7]. In mammals, transcription of genes downstream of hedgehog is upregulated through activation of GLI transcription factors [8]. GLI-2 and GLI-1 are primary activators of the hedgehog signaling pathway, whereas GLI-3 is a repressor of transcription [9]. GLI-2 is required for mouse development, as GLI-2 knockdown mice die prenatally and exhibit defects in the central nervous system [10]. GLI-2 is also shown to be essential for normal prostate development and aberrant activation of GLI-2 is associated with cancers [11]. Previous studies showed that Hh signaling may be correlated with OS [12]. Inhibition of Hh signaling or GLI-2 knockdown in OS cell lines reduces cell proliferation and enhances apoptosis. However, these results did not show detailed investigation of molecular mechanisms.

Aberrant activation of Wnt-β-catenin signaling pathway has been reported to play an important role in tumorigenesis of several cancers such as non-small-cell lung cancer, colorectal carcinoma and so on [13]. Previous studies have demonstrated that the major molecular components of Wnt pathway are detected in OS cells/samples and abnormal activation of the Wnt signaling plays a role in OS pathogenesis

[14]. However, a recent study showed that the Wnt/β-catenin pathway was inactivated in OS samples and that its activation in OS cells inhibited proliferation and induced differentiation [15]. So, the loss of Wnt/β-catenin activity contributed to OS development are still controversial.

Here, we demonstrated that expression levels of GLI-2 increased in osteosarcoma cell lines as revealed by QPCR and western blot compared with normal osteoblast cells. we suggested that crosstalk of Hh/GLI-2-Wnt/β-catenin signaling promotes human osteosarcoma progression *in vitro*. Knockdown of GLI-2 in human osteosarcoma decreased the protein level of β-catenin, further study suggested β-catenin was downstream effector of GLI-2 in promoting proliferation of OS cells. We propose that GLI-2 is a key regulator between Hh/GLI-2-Wnt/β-catenin signaling cross-talk and provides insights into its role in human osteosarcoma progression.

Materials and methods

Cell culture

Human osteosarcoma cell lines U2OS, SaOS cells were maintained in RPMI 1640 supplemented with 10% FBS (GIBCO) plus 100-units/ml penicillin, and 100 mg/ml streptomycin. Human osteoblast cells HOB-c was purchased and cultured in osteoblast growth medium with 50 ml Supplement Mix (PromoCell, Heidelberg, Germany). All the cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. Trypsin (0.25%) was used to detach the cells from the culture flask.

Real-time PCR

Total RNA was extracted from cells using Trizol (Invitrogen). Then, cDNA samples were prepared by using PrimeScript RT reagent Kit (Takara). The cDNA was quantified with the CFX96 system (Bio-Rad) using SYBR Green (Takara). Primer pairs are: GLI-2-F: 5'-GACACCAGGAAGGAAGG-3', GLI-2-R: 5'-GAACGGAGGTAGTGCTCC-3'; GAPDH-F: 5'-GGTGAAGGTCGGAGTCAACGG-3', GAPDH-R: 5'-GAGGGATCTCGCTCCTGGAAG-3'. The expression of GAPDH was used for normalization.

siRNA and transfection

Human GLI-2 siRNA (sc-37913), β-catenin siRNA (sc-29209) and scramble siRNA oligonucleotides (sc-37007) were purchased from Santa Cruz Biotechnology. Transfections were performed with Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions.

Western blotting

Total protein was extracted from cells using RIPA lysis buffer, the protein extracts were denatured by boiling at 95°C for 5 min and equal amounts of proteins were loaded on 10% SDS-PAGE gels and transferred to Nitrocellulose membranes (EMD Millipore). Blots were blocked with 5% non-fat dry milk and then incubated with primary antibodies at 4°C overnight. The primary antibodies included anti-GLI-2 (1:100), anti-β-catenin (1:200, Santa Cruz Biotechnology, and anti-β-actin (1:100, Cell Signaling Technology) antibodies. β-actin was used as the loading control. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse (1:2,000) or anti-rabbit (1:5,000) secondary antibodies at room temperature for 1 h. The blots were developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.).

Proliferation assay

The number of viable cells was assessed using an MTS formazan generation method (Cell Titer-96 Aqueous One Solution Cell Proliferation Assay; Promega, Madison, USA). Cells were treated as indicated and then cells were seeded at a density of 2000 cells per well in 96-well flat-bottom plates. The MTS assay was performed according to the manufacturer's instructions and absorbance was measured at 490 nm.

Statistical analysis

Statistical analyses were performed using SPSS Statistical Package version 16 and all data were expressed as mean ± standard error of mean (SEM). Independent two group's analyses were used for Student's t-test. $P < 0.05$ was considered significant.

Results

Aberrant expression of Hh signaling molecules in osteosarcoma cell lines

To assess the expression of Hh signaling molecule GLI-2 in the progression of OS, we isolated

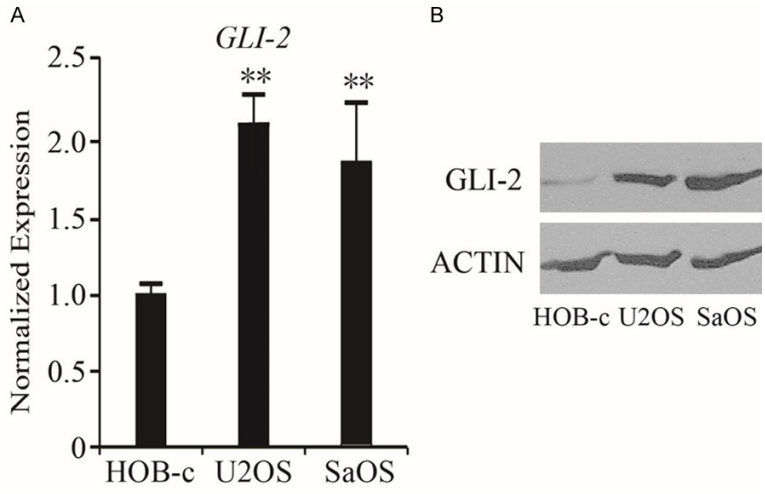

Figure 1. Increased GLI-2 expression in OS cell lines. A. Total RNA was extracted from cells, then RT-QPCR assay was performed. GLI-2 expression was increased in both U2OS and SaOS cells compared with HOB-c cells. **P < 0.01. B. Western blot showed GLI-2 expression was significantly increased in both U2OS and SaOS cells compared with HOB-c cells.

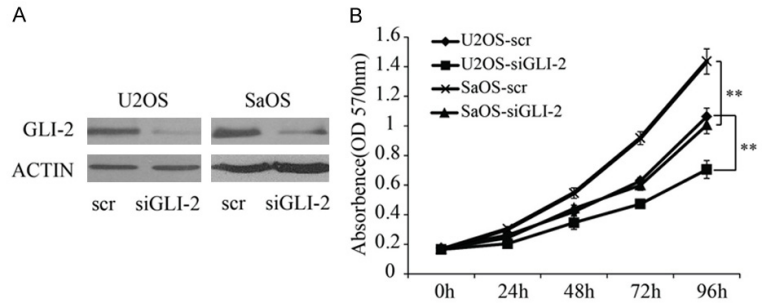

Figure 2. GLI-2 Knockdown suppresses OS cell proliferation. A. Western blot showed GLI-2 expression was significantly decreased after siGLI-2 transfection. B. MTS assay showed siGLI-2 decreased the proliferation of U2OS and SaOS cells in a dose-dependent manner, respectively. **P < 0.01.

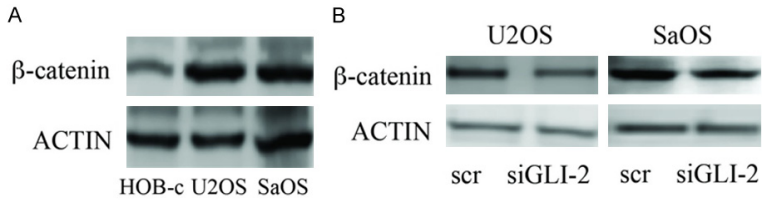

Figure 3. Protein level of β-catenin decreased in GLI-2 knockdown cells. A. Western blot showed β-catenin expression was significantly increased in both U2OS and SaOS cells compared with HOB-c cells. B. Western blotting showed β-catenin expression was significantly decreased in siGLI-2 cells.

total RNA from non-tumorigenic human osteoblast cell line HOB-c and two human osteosarcoma cell lines U2OS, SaOS and prepared cDNA. QPCR results showed that the expression of GLI-2 at mRNA level decreased in both U2OS, SaOS cell lines (**Figure 1A**). We also lysed the cells and detected GLI-2 protein level by western blotting. The expressions of GLI-2 at protein level in OS cell lines were significantly higher than non-tumorigenic osteoblast cell line HOB-c. These results demonstrated that expression of GLI-2 decreased in OS cells.

GLI-2 knockdown by siRNA decreased OS cell proliferation

To explore the effects of GLI-2 on osteosarcoma progression, cell proliferation was analyzed in human OS cell lines U2OS and SaOS. Cells were transfected with siRNA targeting GLI-2 (siGLI-2) for 48 hrs., using scramble siRNA (scr) as control, western blotting showed siGLI-2 efficiently reduced GLI-2 protein expression in both U2OS and SaOS cells (**Figure 2A**). MTS proliferation assay results showed GLI-2 knockdown resulted in decrease of proliferation of U2OS and SaOS cells respectively (**Figure 2B**).

The protein level of β-catenin was decreased in GLI-2 knockdown cells

Because of the crucial role of Wnt signaling in OS progression, the protein level of Wnt signaling marker β-catenin in U2OS and SaOS cells was analyzed. Western blotting results showed the protein levels of β-catenin were increased in U2OS and SaOS cells (**Figure 3A**). Interestingly, we found the protein level of β-catenin was decreased in siGLI-2 transfected U2OS and SaOS cells (**Figure 3B**). These data demonstrated that GLI-2 might

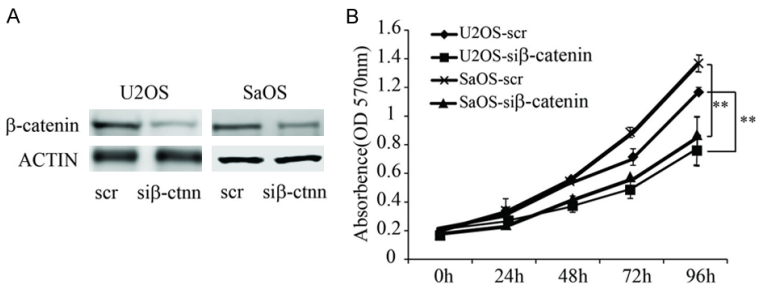

Figure 4. β-catenin Knockdown suppresses OS cell proliferation. A. Western blot showed β-catenin expression was significantly decreased after siβ-catenin transfection. B. MTS assay showed siβ-catenin decreased the proliferation of U2OS and SaOS cells respectively. **P < 0.01.

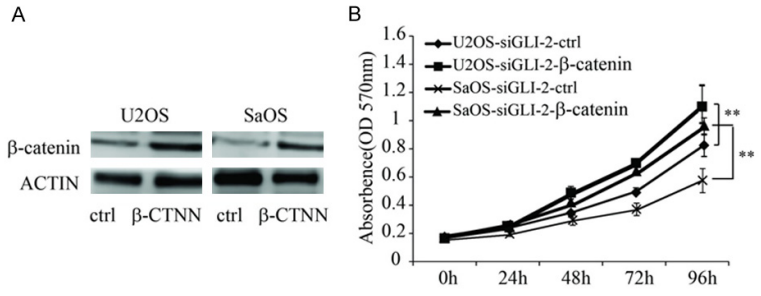

Figure 5. Overexpressioin of β-catenin partially increases proliferation of GLI-2 knockdown cells. A. Western blot showed β-catenin expression. B. MTS assay showed overexpressioin of β-catenin partially increase proliferation of GLI-2 knockdown U2OS and SaOS cells respectively. **P < 0.01.

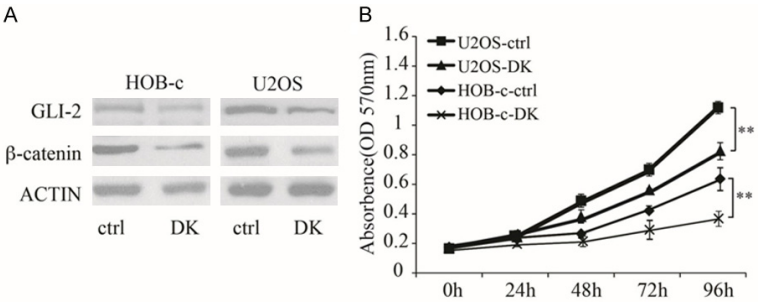

Figure 6. Double knockdown of GLI2 and β-catenin decreases proliferation of both osteoblast and osteosarcoma cells. A. Western blot showed GLI2 and β-catenin expression. B. MTS assay showed double knockdown of GLI-2 and β-catenin decreased proliferation of HOB-c cells and U2OS cells respectively. DK: double knockdown. **P < 0.01.

interact with Wnt signaling to promote proliferation of OS cells.

Knockdown of β-catenin decreased proliferation of OS cells

To explore the effects of β-catenin in osteosarcoma progression, cell proliferation was analyzed in human OS cell lines U2OS and SaOS. Cells were transfected with siRNA targeting β-catenin (siβ-catenin) for 48 hrs., using scramble siRNA (scr) as control, western blotting showed siβ-catenin efficiently reduced β-catenin protein expression in both U2OS and SaOS cells (**Figure 4A**). MTS proliferation assay results showed β-catenin knockdown resulted in decrease of proliferation of U2OS and SaOS cells respectively (**Figure 4B**).

Overexpression of β-catenin in GLI-2 knockdown cells increased proliferation of OS cells

To further study whether β-catenin is involved in the GLI-2 regulated proliferation of OS, we overexpressed β-catenin (**Figure 5A**) in U2OS and SaOS cells. The MTS results showed overexpression of β-catenin partially increased the proliferation rate of GLI-2 knockdown cells (**Figure 5B**).

Knockdown of β-catenin and GLI-2 further decreased proliferation of both osteoblast and OS cells

To further study the role of β-catenin and GLI-2 in regulation of proliferation of OS, we depleted GLI2 and β-catenin together (double knockdown, DK cells) using siRNA (**Figure 6A**) in HOB-c (HOB-c-DK) and U2OS (U2OS-DK) cells and compared the proliferation between HOB and osteosarcoma. The MTS results showed that, compared with cells treated with scramble siRNA, the proliferation rate of both HOB-c-DK cells and U2OS-DK cells decreased dramatically (**Figure 6B**).

Discussion

The HH and WNT pathways play pivotal roles in development and in a number of cancers including osteosarcoma [12, 16, 17]. Chan et al. showed that upregulated Hh signaling in

mature osteoblasts significantly induces osteoblastic osteosarcoma [12]. Nagao et al. showed that GLI2 is highly expressed in human osteosarcoma samples [10]. Tabatabai et al. reviewed the function of Wnt pathways in osteosarcoma progression [13], showing aberrant activation of Wnt/β-catenin pathway is closely associated with the progression of osteosarcoma. Recently, Song et al. showed crosstalk between Wnt/β-catenin and Hedgehog/Gli signaling pathways play critical roles in colon cancer [17]; however, the crosstalk between these two pathways has not been fully investigated in human osteosarcoma progression. Here we provide evidence of HH/GLI-2 crosstalk with the WNT/β-catenin pathway in human OS cell lines and the role of GLI-2 in tumorigenesis of OS.

Our results demonstrate that, compared to hFOB, the expression of GLI-2 which is a transcriptional effector of HH signaling pathway was upregulated in both U2OS and SaOS cells. Knockdown of GLI-2 by siRNA showed GLI-2 is necessary to promote OS cell proliferation.

β-catenin, the downstream mediator of the Wnt pathway, is usually confined to the cell membrane and its nuclear relocalization and transcriptional activity is tightly regulated [18]. Activation of β-catenin is strongly implicated in tumorigenesis and metastasis [19]. We provide evidence of upregulation of β-catenin in both U2OS and SaOS cells. We also showed that knockdown of β-catenin decreased the proliferation of OS cell lines.

Interestingly, decrease of expression of β-catenin was observed in siGLI-2 cells suggesting that β-catenin expression in human OS cells may be regulated by GLI2 and that β-catenin can be a downstream effector of the HH/GLI-2 pathway. Our further study proved that overexpression of β-catenin in GLI-2 knockdown cells partially increased proliferation of OS cells.

Furthermore, we showed that double knockdown of β-catenin and GLI-2 decreased proliferation of both osteoblast and OS cells, suggesting crosstalk between HH and WNT pathways play pivotal roles in both osteoblast cells and OS cells.

In conclusion, we show that GLI-2 is an upstream regulator of β-catenin in human OS cell lines and that its expression promotes the proliferation of OS cell lines partially through cross talk with Wnt/β-catenin signaling pathway. Altogether, this evidence provides valuable insights into the role of HH and Wnt signaling pathway in tumorigenesis of OS. Knowledge of the HH/GLI-2-WNT/β-catenin crosstalk may also help to identify potential new targets for therapy.

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Disclosure of conflict of interest

None.

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