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Original Article

Inhibition of WNT7A-β-catenin signaling pathway sensitizes oral squamous cell carcinoma to cisplatin

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Abstract: Oral squamous cell carcinoma (OSCC) is the most common type and most threatening head and neck cancer worldwide. Here, we aim to study the relationship between the WNT7A-β-Catenin signaling pathway and the chemotherapy resistance of OSCC patients. We analyzed 42 OSCC patients and 19 adjacent non-tumor tissues, evaluated the expression levels of WNT7A mRNA, and subsequently studied WNT7A dependent cisplatin resistance in OSCC cell line KB cells. Moreover, we also utilized an *in vivo* mouse model to validate our findings. We first found a significant upregulation of WNT7A mRNA in OSCC patients. Our results showed that the knockdown of WNT7A sensitized KB cells to cisplatin. Moreover, our results revealed that nuclear β-catenin was dramatically reduced and cleaved caspase-3 and cleaved PARP were dramatically induced when WNT7A was knocked down in cisplatin treated KB cells. Besides, we found that the knockdown of WNT7A significantly reduced the weight and volumes of xenograft tumors. Moreover, we examined apoptotic cells and found that the combination of WNT7A knockdown and cisplatin treatment resulted in many more apoptotic cells than cisplatin treatment alone, suggesting that the knockdown of WNT7A sensitized KB cells to cisplatin treatment *in vivo*. Our results revealed that inhibition of WNT7A-β-catenin signaling sensitizes OSCC to cisplatin, which has provided insights into the molecular diagnosis and treatment of OSCC.

Keywords: WNT7A, β-Catenin, OSCC, cisplatin, drug resistance

Introduction

Oral squamous cell carcinoma (OSCC) accounts for over 90% of all oral cancers and is the most common type of head and neck cancer worldwide [1, 2]. Although advances have been made to improve the therapies for OSCC, overall survival has not been correspondingly ameliorated [3]. Although it has been demonstrated that chemotherapy increased survival rates [4], cancers with various genetic backgrounds respond differently to chemotherapy, and tumors under continuous chemotherapy treatment tend to develop resistance [5, 6]. However, the mechanisms of chemotherapy resistance in OSCC is far from clear. Thus, it is critical to identify the genetic backgrounds that affect the sensitivity of OSCC to first line chemotherapy. Cisplatin is one of the most common and economical chemical compounds that has been modified for anticancer treatment, and it has been long used for the treatment of OSCC. However, a large proportion of OSCC patients develop chemo-resistance to cisplatin [7].

Accumulating evidence suggests that cancer stem cells play crucial roles in the development of drug resistance, and it has been identified that β-Catenin, a central factor in stem cell biology, induces cisplatin resistance in OSCC [8, 9]. Wnt/β-Catenin signaling is a key regulator in the proliferation and differentiation of stem cells as well as epithelial cells [10]. Importantly, the Wnt/β-catenin pathway is also involved in various types of cancers [11]. In all, there are 19 cysteine-rich Wnt proteins that regulate the Wnt/β-catenin pathway in various biological process [12]. The expression of Wnt ligands has been characterized in ameloblastoma [13], suggesting that the Wnt/β-catenin pathway is critical in oral tumors. Wnt7b was reported to promote the proliferation and invasion of OSCC by regulating theWnt/β-catenin signaling and WNT7A was found to be silenced in mesenchy-

mal-like OSCC cell lines [14, 15]. However, the precise role of WNT7A in OSCC is unclear.

WNT7A was identified as an important regulator in dorsoventral pattern establishment in the vertebrate limb [16]. The altered expression pattern of WNT7A was identified in human lung cancer, and it was then found to be upregulated in colorectal cancer, pancreatic cancer and gastric cancer cell lines [17], suggesting that WNT7A may have a role in promoting the pathology of tumor development. Interestingly, WNT7A was found to have a pro-proliferation function in stem cells by activating the planar cell polarity pathway [18]. Recent reports also have supported the revelation that WNT7A is actively involved in various types of cancers, such as clear cell renal cell carcinomas, endometrial carcinomas and ovarian cancers [19-23]. Thus, it is important to reveal the role of WNT7A in OSCC which would provide insights for diagnosing and treating OSCC.

A recent report showed that WNT7A was secreted by aggressive breast cancer cells which in turn induces the conversion of cancer-associated fibroblasts [24], further demonstrating a pro-cancer role of WNT7A. More importantly, results from two groups independently indicated that WNT7A is a predictor of chemosensitivity in malignant pleural mesothelioma and ovarian cancer respectively [25, 26]. King et al., found that WNT7A increases the sensitivity of ovarian cancer cells to niclosamide [26], suggesting that the WNT7A/β-catenin signaling controls the sensitivity of cancer cells to anticancer chemical compounds. Thus, it is worth exploring whether WNT7A is participated in the chemoresistance of OSCC.

In the present study, we show that the expression of WNT7A is highly up-regulated in OSCC patient samples and in the mouse cancer cell line KB. Our results show that the existence of WNT7A antagonizes cisplatin treatment, causes the loss of WNT7A inhibited β-catenin activity and induces apoptosis. Moreover, our *in vivo* data further suggest that the downregulation of WNT7a sensitizes OSCC to cisplatin chemotherapy.

Materials and methods

Patient samples and cell culture

42 OSCC tissues and 19 adjacent normal tissues in this study were obtained from patients with OSCC who underwent surgery in the Second Affiliated Hospital of Xi'an Jiaotong University after receiving their written consent. The use of human tissue samples was approved by the Institutional Review Boards of Xi'an Jiaotong University. Human oral epithelial carcinoma cell line KB was purchased from ATCC (Manassas, VA, USA). KB cell was cultured in an RPMI medium 1640 (Gibco, USA), supplemented with 10% FBS (Hyclone, USA), and 1% penicillin/streptomycin (Gibco, USA). All cells were incubated at 37°C with 5% CO_2.

RNA extraction and RT-qPCR

Total RNA of tissues and cells were extracted using the TRIzol (Invitrogen, USA) reagent according to the manufacturer's instructions. RNAs were reversely transcribed into cDNAs using the Transcriptor First-Stand cDNA Synthesis Kit (Roche Molecular Biochemicals, Germany) according to the manufacturer's instructions. RT-qPCR was performed using an ABI 7900 system (Applied Biosystems, USA) to detect relative expression levels of WNT7a and GAPDH. The reaction conditions were set as follows: 40 cycles of pre-denaturation at 95°C for 10 min, denaturation at 90°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 34 s. The 2-ΔΔCt method was used to calculate the relative expression of WNT7a, GAPDH was used as the controls. The results are representative of three independent experiments. The primers are as follows: WNT7a: forward primer: 5'-CAAAGAGAAGCAAGGCCAGTACCA-3', reverse primer: 5'-GTAGCCCAGCTCCCGAAACTGT-3'; GAPDH: 5'-GGTTTACATGTTCCAATA-3', reverse primer: 5'-ATGGGATTTCCATTGATGACAAG-3'.

Western blot analysis

Cells were lysed in a RIPA buffer (Beyotime Biotechnology, China) containing the protease inhibitor cocktail (Sigma). The nuclear fraction of KB cells was isolated using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, China). After quantifying protein concentrations with a BCA Protein Assay Kit (Beyotime Biotechnology, China), equal amounts of proteins were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, USA). The membrane was blocked in 5% nonfat milk for 1 hour, and the proteins were probed with indicated primary antibodies overnight at 4°C. The antibodies are as follows: anti-WNT7a (Abcam, ab-

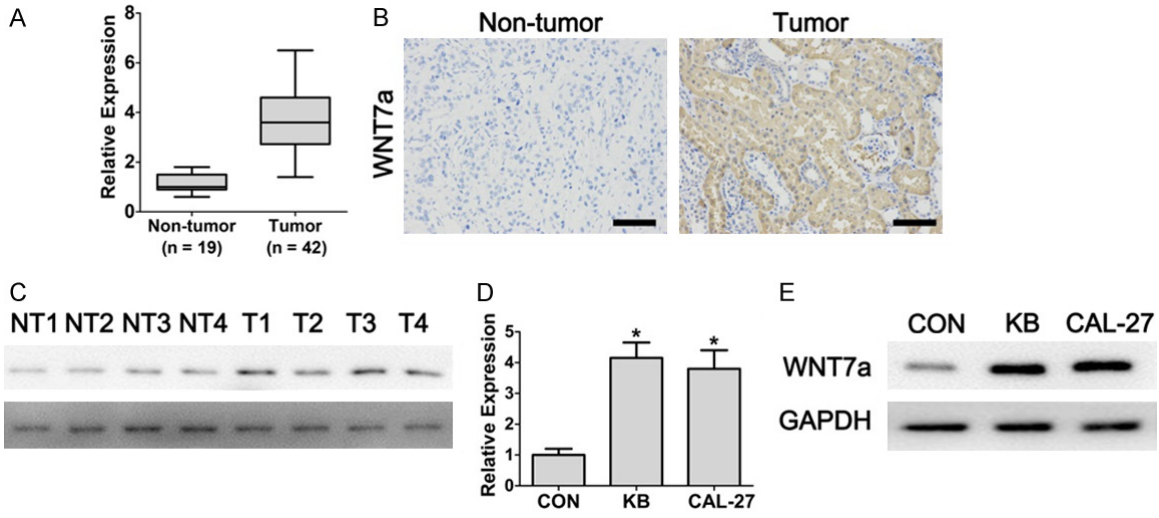

Figure 1. WNT7a is highly expressed in oral squamous cell carcinoma (OSCC). (A) qPCR analysis showed WNT7a was upregulated in 42 OSCC tissues compared to 19 normal tissues. (B) Immunohistostaining of WNT7a in OSCC patients. Non-tumor, adjacent normal tissues; tumor, cancerous tissues from OSCC patients. (C) Western blot analysis of WNT7a in OSCC patients. NT, non-tumor; T, tumor. qPCR (D) and Western blot (E) analysis of WNT7a in human oral cancer cell line KB and primary mucosal epithelial cell (CON). GAPDH was used as normalization control. The experiments were repeated at least three times. *, $P < 0.05$ compared to control. Values represent mean ± SD.

217844, 1:500), anti-GAPDH (Abcam, ab8245, 1:1000), anti-Histone H3 (Abcam, ab13847, 1:1000), anti-active β-catenin (Millipore, 05-665, 1:300), anti-Caspase-3 (Abcam, ab13847, 1:500), anti-PARP (Abcam, ab32138, 1:1000). After washing and incubating with HRP-linked secondary antibodies at room temperature for 2 hours, proteins were detected using an ECL system (Millipore, USA).

RNA interference

Cells were transfected using Lipofectamine 3000 (Invitrogen, USA) with small interfering RNAs targeting WNT7a (Thermo Fisher Scientific, siRNA ID: 121498). A scrambled siRNA was used as a negative control. Transfection was performed following the manufacturer's protocol.

MTT cell proliferation and cytotoxicity assay

Cells were seeded into 96-well culture plates (5 × 10^3 cells/well) and treated with cisplatin in various concentrations: 0.1, 1, and 10 μM for 48 hours, 10 μl MTT reagent (Beyotime Biotechnology) was added to each well and incubated for 2 h. Then we read the absorbance values at a wavelength of 570 nm using a microplate reader (SpectraMax 250; GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Tumor formation assay

3 × 10^6 KB/siScramble cells and KB/siWNT7a cells were prepared and injected subcutaneously into the right and left flanks of 6-week-old athymic nude mice (n = 10). Chemotherapeutic groups were treated with cisplatin (2 mg/kg) by intraperitoneal injection every 3 days and the vehicle control group was injected with an equal volume of DMSO. Xenotransplanted tumors were measured by caliper every day for 6 days, and tumor volume was calculated by the following formula: volume = 0.5 × length × width^2 (length: longest diameter in mm, width: the shortest diameter in mm). The animals were then sacrificed and individual tumor weights were measured. All animal procedures were approved by Institutional Animal Care and Use Committee of Xi'an Jiaotong University.

TUNEL staining

TUNEL staining was performed in KB-xenografted tumor sections as previously described [27]. Briefly, TUNEL staining was performed using the Click-iT Plus TUNEL Assay, Alexa Fluor 488 dye (Thermo Fisher Scientific, C10617) according to the manufacture's protocol. The nuclei were visualized by the Hoechst 33342 stain (Thermo Fisher Scientific, H3570).

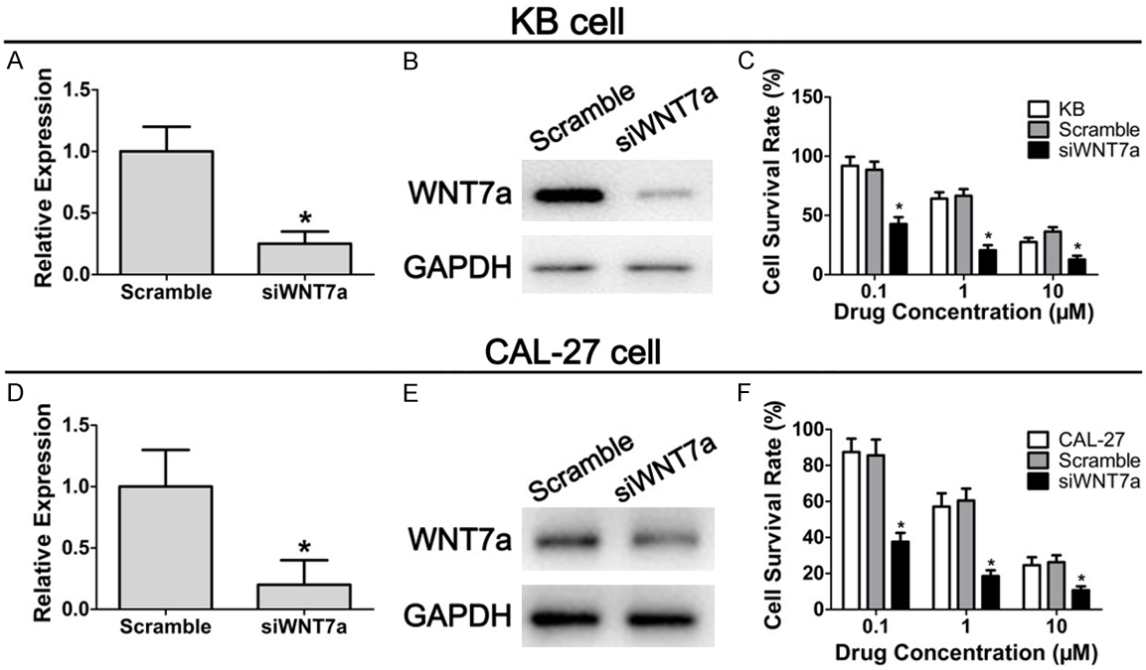

Figure 2. WNT7a knockdown sensitizes KB cells to cisplatin. Forty-eight hours after transfection with WNT7a siRNA or scrambled siRNA, RNA interference efficiency was evaluated by qPCR (A and D) and western blot (B and E) in KB cells and CAL-27 cells repectively. GAPDH was used as a normalization control. (C) WNT7a downregulated KB cells were treated with cisplatin with various concentrations: 0.1, 1, and 10 μM for 48 hours. Cell survival rates were determined by MTT assay. (F) WNT7a downregulated CAL-27 cells were treated with cisplatin with various concentrations: 0.1, 1, and 10 μM for 48 hours. Cell survival rates were determined by MTT assay. *, $P < 0.05$ compared to normal KB cells transfected with scrambled siRNA. Data were collected from three independent experiments. Values represent mean ± SD.

Statistical analysis

All data were expressed as the mean ± SD and were collected from at least three replicates per experiment. Data were analyzed using Student's t-test in microsoft office excel 2010. A value of $p < 0.05$ was considered to be statistically significant.

Results

WNT7A is up-regulated in OSCC

To understand the role of WNT7A in OSCC, we first examined the mRNA levels of WNT7A in OSCC tissues and adjacent normal tissues. In all, we examined the mRNA levels of WNT7A in 42 OSCC patients and 19 adjacent non-tumor tissues. Our results revealed a significant up-regulation of WNT7A in OSCC patients (**Figure 1A**), suggesting that WNT7A plays an important role in OSCCs. To confirm the expression pattern of WNT7A in OSCC tissues, immunohistostaining was conducted. The representative IHC images are shown in **Figure 1B**, and the WNT7A expression was predominantly detected in OSCC tumors. Our Western blot result further validated the upregulated protein level of WNT7A in OSCC tumors (**Figure 1C**). We also analyzed the expression of WNT7A in the human oral cancer cell line KB and in the normal primary mucosal epithelial cell line. In accordance with the findings in the patient samples, both mRNA and protein levels of WNT7A were increased in human oral cancer cells compared to the primary mucosal epithelial cells (**Figure 1D** and **1E**).

WNT7A antagonizes cisplatin treatment of OSCC in vitro

To further explore the function of WNT7A in OSCC, we knocked down WNT7A with siRNAs in KB cells. Our results showed that both the mRNA and protein levels of WNT7A were successfully inhibited at 48 h post-transfection of the siRNAs (**Figure 2A** and **2B**). More importantly, our results showed that the knockdown of WNT7A resulted in dramatic cell death when treated with cisplatin at various concentrations

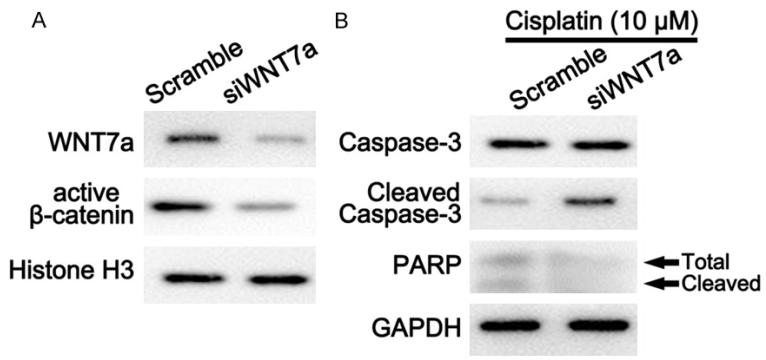

Figure 3. WNT7a knockdown deactivates β-catenin pathway. A. Forty-eight hours after RNA interference, nuclear proteins in KB cells were extracted and analyzed by Western blot, showing that active β-catenin was decreased in WNT7a-downregulated KB cells. Histone H3 was used as a loading control. B. WNT7a knockdown further activates caspase-3/PARP in cisplatin-treated KB cells. KB cells trasfected with WNT7a siRNA or scrambled siRNA were treated with 10 μM cisplatin and total proteins were extracted for Western blot analysis. GAPDH was used as a loading control.

(**Figure 2C**), suggesting that the expression of WNT7A antagonized the cytotoxicity of cisplatin to KB cells.

Loss of WNT7A inhibits β-catenin activity and activates the caspase-3 pathway

Since WNT7A is a typical inducer of β-catenin signaling, we examined the nuclear β-catenin level by extracting the nuclear proteins. Our results showed decreased accumulation of nuclear β-catenin in cells with downregulated WNT7A (**Figure 3A**). It has been reported that β-catenin is regulated by the caspase-3 pathway in apoptotic cells [28]. We next detected caspase-3 and its downstream protein Poly (ADP-Ribose) Polymerase (PARP), our results showed that when treated with 10 μM cisplatin, cleaved caspase-3 and cleaved PARP were dramatically increased in WNT7A downregulated cells compared with the control cells, indicating that the loss of WNT7A leads to the activation of the caspase-3 pathway in apoptotic KB cells. Taken together, these results provide evidence that the downregulation of WNT7A potentiates KB cells to cisplatin treatment by activating Caspase-3, which further deactivates the β-catenin pathway.

Downregulation of WNT7A sensitizes OSCC to cisplatin in vivo

To further demonstrate the function of WNT7A in OSCC, we injected KB cells transfected with WNT7A siRNAs or scramble siRNAs into nude mice. We further divided these mice into two groups: the chemotherapeutic group was intraperitoneally treated with 2 mg/kg cisplatin; the vehicle control group was injected with an equal volume of DMSO. Our results showed that the knockdown of WNT7A significantly reduced the weight and volumes of the tumors (**Figure 4A-C**). In addition, the knockdown of WNT7A remarkably decreased the chemoresistance of KB cells to cisplatin treatment *in vivo*, as evidenced by the further inhibited growth of WNT7A downregulated KB-xenograft after cisplatin treatment (**Figure 4C**). Moreover, our TUNEL assay confirmed that cisplatin treatment resulted in more apoptotic cells than cisplatin treatment alone (**Figure 4D**) suggesting that knockdown of WNT7A sensitized KB cells to cisplatin treatment *in vivo*.

Discussion

More than 4 decades of efforts have been put into improving the overall survival of oral squamous cell carcinoma, but little has been accomplished. About 30% of OSCC deteriorates into untreatable recurrent OSCC [29, 30]. It is an effective routine to identify factors that are specifically altered in OSCC in order to develop customized therapies for OSCC patients. Here, we identified that WNT7A is upregulated in OSCC patients and the KB cell line. WNT7A was previously characterized as a Wnt ligand that facilitates limb development via regulating β-Catenin signals [31]. Recent reports have demonstrated that it plays critical roles in various type of human cancers [23, 32, 33]. Our data examined the expression pattern of WNT7A in OSCC and found that WNT7A was dramatically increased in OSCC, further validating the pro-cancer role of this ligand. β-catenin signaling forms a nexus that governs multiple biological process in stem cells and cancer cells, so it is urgent to understand the molecular mechanism of the WNT7A-β-Catenin axis in regulating cancer cells [11].

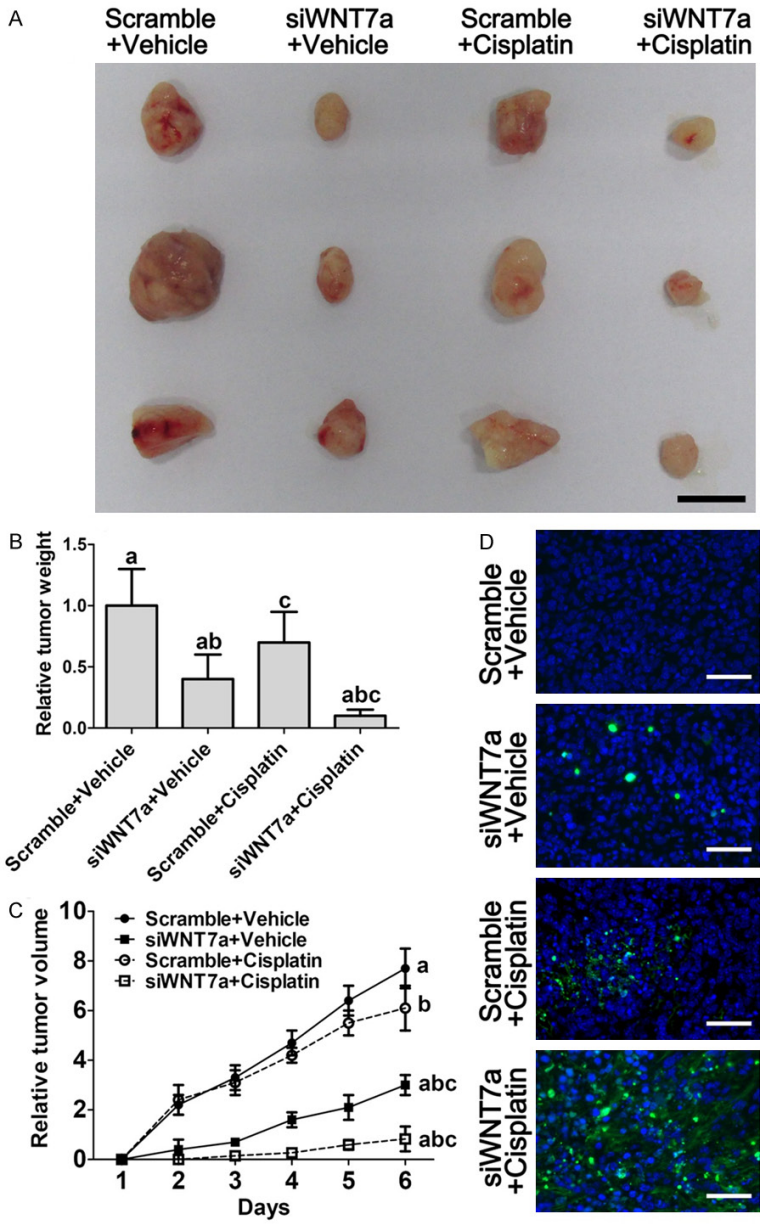

Figure 4. Downregulation of WNT7a sensitizes OSCC to cisplatin *in vivo*. Athymic nude mice were injected subcutaneously with 3 × 10^6 WNT7a-downregulated (siWNT7a+Vehicle and siWNT7a+Cisplatin) or control cells (Scramble+Vehicle and Scramble+Cisplatin). Chemotherapeutic groups were treated with cisplatin (2 mg/kg) by intraperitoneal injection every 3 days. (A) Representative overview of KB-xenografted tumors. Scale bar, 1 cm. Tumor weight (B) and volume (C) were decreased in WNT7a-downregulated mice. Statistical analysis was performed using Student's t test; significant differences were shown in groups sharing the same letter. (D) Intratumoral apoptosis was analyzed by TUNEL assay. Scale bars, 50 μm.

Interestingly, when WNT7A is knocked down in KB cells, the cancer cells became more sensitive to cisplatin, a first-line treatment for OSCC [34]. And importantly, this alteration of drug sensitivity was under the environment of reduced β-Catenin activity (**Figure 3A**). Previous reports have indicated that the over expression of β-catenin in OSCC induced cisplatin resistance *in vitro* and *in vivo* [9] supporting our results that the knockdown of WNT-7A, which inhibited β-Catenin activity, would increase the sensitivity of OSCC to cisplatin. VEGF signaling, the glutathione pathway, and p22phox have also been suggested as playing a role in affecting cisplatin sensitivity in squamous cell carcinomas [35, 36]. Interestingly, Oct4 and NANOG, another two critical factors in stem cell biology, also showed correlations with cisplatin resistance in oral squamous cell carcinoma [37], suggesting that cancer stem cells are vital in the pathology of OSCC.

Our results showed that cleaved Caspase-3 and cleaved PARP were dramatically increased when WNT7A knocked down KB cells were treated with cisplatin, indicating that apoptosis was induced when WNT7A knocked down KB cells were treated with cisplatin (**Figure 3B**). A previous report showed that reduced WNT7A and nuclear β-Catenin resulted in the induction of the cleavage of caspases and PARP, subsequently causing apoptosis [38]. Our data further validated that the loss of WNT7A would induce the cleavage of caspases and PARP, as well as apoptosis in OSCC, indicating that the existence of WNT7A inhibited cisplatin-induced apoptosis. Also, PG545, a heparan sulfate mimetic, was shown to interact with WNT7A and block β-Catenin signaling which, in the end, inhibited pancreatic tumor cell proliferation and induced pancreatic tumor cell apoptosis [39]. However, it has also been suggested that WNT7A is a β-Catenin independent tumor suppressor that promotes

lung cancer cell senescence [33]. These controversial results need further validation. Moreover, our results provided *in vivo* evidence that the combination of WNT7A knockdown and cisplatin treatment significantly inhibited tumor growth and increased apoptosis (**Figure 4**), which further indicates that WNT7A is a potential target to increase chemosensitivity in OSCC.

In summary, our results are the first evidence that WNT7A is upregulated in OSCC patients and in cell lines. Furthermore, the knockdown of WNT7A sensitized OSCC to cisplatin. More importantly, combination of WNT7A knockdown and cisplatin treatment resulted in a significant decrease of tumor weights and volumes via increasing apoptosis. Our results revealed novel molecular mechanisms of WNT7A-dependent drug resistance which provide insights into the molecular diagnosis and treatment of OSCC.

Acknowledgements

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Disclosure of conflict of interest

None.

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