

Original Article

TCAB1 knock-down regulates the Wnt/ β -catenin signaling pathway and affects cancer stem cell properties in oral squamous cell carcinoma

Mengwen Zheng*, Chao Guo*, Jiang Xu, Changxue Li

Department of Stomatology, The First Affiliated Hospital, School of Medicine, Shihezi University, Shihezi, Xinjiang Uygur Autonomous Region, China. *Equal contributors and co-first authors.

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Abstract: Objective: To study the regulatory mechanism of Telomerase Cajal Body Protein 1 (TCAB1) gene silencing on the biological characteristics of oral squamous cell carcinoma tumor stem cells through the Wnt/ β -catenin signaling pathway. Methods: Tumor stem cells in oral squamous cell carcinoma cells were sorted using magnetic beads. The tumor stem cells were split into five groups: the blank group, the negative control group (the NC group), the si-TCAB1 group, the Wnt/ β -catenin pathway inhibitor group (the XAV939 group), and the si-TCAB1 + Wnt/ β -catenin pathway inhibitor group (the si-TCAB1 + XAV939 group). The mRNA and protein expressions of TCAB1, Wnt3a, β -catenin, caspase 9, B-cell lymphoma-2 (Bcl-2) and BCL2-Associated X (Bax) were investigated using qRT-PCR and Western Blot. A methyl thiazolyl tetrazolium (MTT) assay, a Transwell test, and flow cytometry were used to measure the cell proliferation activity, apoptosis, and invasion, respectively. Results: Compared with the blank group and the NC group, the TCAB1 was expression significantly decreased in the si-TCAB1 and the si-TCAB1 + XAV939 groups ($P < 0.05$), and there were reduced Wnt3a, β -catenin and Bcl-2 expressions, increased caspase 9 and Bax expressions, the cell proliferation activity and invasion were decreased, and the cell apoptosis rate was raised in the si-TCAB1, XAV939, and si-TCAB1 + XAV939 groups (all $P < 0.05$). The blank and NC groups showed no significant differences (all $P > 0.05$). Compared with the si-TCAB1 group, there was increased TCAB1 expression in the XAV939 group ($P < 0.05$) but there were no differences in the other indicators (all $P > 0.05$), and there were decreased Wnt3a, β -catenin and Bcl-2 expressions, increased caspase 9 and Bax expressions, decreased cell proliferation activity and invasion, and a higher cell apoptosis rate in the si-TCAB1 + XAV939 group (all $P < 0.05$). Conclusion: TCAB1 gene silencing can inhibit the activation of the Wnt/ β -catenin signaling pathway, thereby arresting the proliferation and invasion of the oral squamous cell carcinoma tumor stem cells and promoting apoptosis.

Keywords: TCAB1 gene, oral squamous cell carcinoma, Wnt/ β -catenin signaling pathway, tumor stem cell

Introduction

Oral squamous cell carcinoma (OSCC) is a common malignant tumor in the oral and maxillofacial region with high recurrence and metastasis rates [1]. The infinite renewal and multiple differentiation of cancer stem cells (CSCs) in the formation and progression of various tumors, including OSCC, have been increasingly reported, and they are the fundamental cause of distant metastasis and the expansion of tumors [2, 3]. Therefore, the exploration of CSCs is significant in clarifying tumors' development, progression, and drug resistance.

Telomerase has been proved to be highly expressed in tumors and promote tumor progres-

sion [4-6]. Telomerase Cajal Body Protein 1 (TCAB1), also known as WD-repeat protein 79 (WDR79), is a core protein of telomerase, and it plays an important role in the function of telomerase and is also essential for telomere synthesis in human cancer cells [7, 8]. TCAB1 is closely associated with non-small cell lung cancer (NSCLC) and nasopharyngeal carcinoma (NPC) [9, 10]. In addition, the level of TCAB1 is increased in head and neck cancer, and its inhibition hinders tumor formation in nude mice in vitro [11]. However, the underlying mechanism of TCAB1 and its association with OSCC remain largely unknown. The core components of the canonical Wnt signaling pathway include ligands, transmembrane receptors, nuclear tran-

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scription factors, and cytoplasmic proteins (β -catenin and GSK-3 β). The binding of Wnt to transmembrane receptor enhances the interaction between the receptor and cytoplasmic proteins and increases nuclear accumulation of β -catenin. Then β -catenin interacts with nuclear transcription factors and activates downstream target gene transcription, thus making the Wnt signaling pathway exert its biological role [12]. It had been reported that the Wnt pathway is involved in the progress of various human diseases [13, 14], wherein the Wnt/ β -catenin signaling pathway is a key pathway in the progression of OSCC [15]. Cancer cells with the potential of self-renewal and unlimited proliferation are called cancer stem cells (CSCs) [16]. CSCs share many properties with normal stem cells, for example, two division modes, telomerase activity and telomeric repeat sequences, similar markers, and growth regulating signaling pathways (Wnt and Notch) [17]. The involvement of TCAB1 in tumors is achieved by regulating the MDM2-p53 signaling pathway [18]. In addition, another member of the WDR family, WDR59, has been found to affect the Wnt signaling pathway [19]. However, studies on whether TCAB1 affects the progression of OSCC by regulating the Wnt pathway have not been carried out.

Therefore, this paper focuses on this issue from the perspective of the effects of CSCs on the pathogenesis of OSCC and explores the related mechanisms.

Methods

Cell culture

CAL-27 cells (human OSCC cell line, Huiying Biotechnology Co., Ltd., China) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS, C22400500BT, Shanghai Huiying Biotechnology Co., Ltd., China) at 37°C including 5% CO₂. When they reached 80%-90% confluence, the cells were digested and subcultured with 0.25% trypsin (252000056, Thermo Fisher, USA).

Isolation of the CSCs using magnetic-activated cell sorting (MACS)

Auto MACS Pro (Miltenyi Biotechnology Co., Ltd., Germany, 130-092-545) was employed to isolate the CD133⁺/CD44⁺ cells (CSCs) [20-22].

The CAL-27 cells were trypsinized, centrifuged, counted, and transferred to centrifuge tubes ($\sim 3 \times 10^7$ cells/mL). Next, the cells were resuspended with phosphate buffer saline (PBS), 20 μ L of mouse anti-human CD133 polyclonal antibody (1:300, ab19898, Abcam, UK) was added, and the final concentration was 15 μ L/mL, then we incubated the mixture at 4°C for 30 min and centrifuged it at 1000 r/min for 5 min. After removing the supernatant, 20 μ L of immunomagnetic beads covered with sheep anti-mouse IgG (YB-22160, Shanghai Yubo Biotechnology Co., Ltd., China) with a concentration of 4×10^8 cells/mL were added, and we incubated the mixture for 15 min at room temperature. The test tubes were placed on a magnetic separator and allowed to stand for 3 min. The supernatant (CAL-27 rich in CD133⁻) was removed. A DNase buffer was applied to separate the CD133⁺ cells from the magnetic beads, and the test tubes were placed on the magnetic separator for 1 min so that the beads were adsorbed on the wall of the tube close to the separator. The supernatant rich in CD133⁺ cells was re-suspended into the cell suspension (2×10^8 cells/mL) and mixed with mouse anti-human CD44 monoclonal antibody (1:500, ab157107, Abcam, UK) and the final concentration was 15 μ L/mL. Next, we incubated the mixture at 4°C for 30 min and centrifuged it at 1000 r/min for 5 min. The supernatant was discarded, and a 2×10^8 cells/mL resuspension was prepared. Then 20 μ L of immunomagnetic beads covered with sheep anti-mouse IgG (4×10^8 cells/mL) were added to the resuspension. The mixture was then incubated for 15 min at room temperature. Once again, the test tubes were placed on a magnetic separator and allowed to stand for 3 min. After removing the supernatant (CAL-27 rich in CD44⁻), a DNase buffer was applied to separate the CD133 (CD44⁺ cells) from the magnetic beads, and the test tubes were placed on the magnetic separator for 1 min. The supernatant rich in CD44⁺ cells was collected when the magnetic beads were adsorbed on the wall of the tube close the separator. The isolated cells were cultured in RPMI 1640 medium containing 10% FBS, 20 ng/mL recombinant human epidermal growth factor (EGF), and 10 ng/mL recombinant human basic fibroblast growth factor (bFGF) at a constant 37°C and 5% CO₂. Then the purity of the CD44⁺ and CD133⁺ cells was measured using flow cytometry.

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Table 1. qRT-PCR Primer sequences

| Gene | Primer sequences (5'-3') |
|-----------|--------------------------|
| Tcab1 | |
| Forward | GAAAACTGGATGATGTCATCCGC |
| Reverse | TTAAGCCTGCCTCTGGGTAAC |
| Wnt3a | |
| Forward | TTGTGGACGGGGAAGTTAGCGA |
| Reverse | GGTAGCGGAATGTGTTAGGTGGTA |
| B-catenin | |
| Forward | CCCTCCACACTGAGATCGC |
| Reverse | CCACTCACCCTCTGAGCG |
| Caspase 9 | |
| Forward | CTCGGCGATTCTTAACATACTGG |
| Reverse | GCTAGGTGGACCACGGTATT |
| Bax | |
| Forward | CGCGAGTTTTCCGCTCAGTAG |
| Reverse | CAGCGGTTTCACATTGACCGT |
| Bcl-2 | |
| Forward | GGGGTCAGGTGTGTGTGG |
| Reverse | CTTCGTTTCAGTCACATGAGGCC |
| Gapdh | |
| Forward | TGTTGGGCATCAAATTGGTGG |
| Reverse | ACCCATCTGTACGGGTCAATAT |

Note: TCAB1: telomerase Cajal body protein 1; Bax: BCL2-Associated X; Bcl-2: B-cell lymphoma-2; qRT-PCR: Quantitative real time polymerase chain reaction.

siRNA sequences

The sequences of the small interfering RNA (siRNA) interfering TCAB1 were supported by the Shanghai Gene Pharma Technology Co., Ltd. Each siRNA pair consisted of two end-flapped complementary oligonucleotides. TCAB1-siRNA sequence: 5'-GUGUGGGUGAGUU-GAUUATT-3', 5'-UAUAUCAGCUCACCCACACTT-3', negative control sequence: 5'-UUCUCC-GAACGUGUCACGUTT-3', 5'-ACGUGACACGUUC-GGAGAATT-3'.

Cell grouping and transfection

The sorted CSCs were divided into 5 groups: the blank group (CAL-27 cells receiving no transfection), the NC group (CAL-27 cells transfected with the NC plasmid), the si-TCAB1 group (transfected with si-TCAB1), the pathway inhibitor group (transfected with inhibitor of Wnt/ β -catenin signaling pathway XAV939 after cell isolation, concentration: 30 μ mol/L), and the combination group (transfected with si-TCAB1 and XAV939). Transfection methods: siRNA

was added into RNase-free H₂O to prepare a liquid with a concentration of 20 μ M. The cells were inoculated into 6-well plates with 1×10^5 cells per well 24 h before the transfection and then cultured in complete medium. After the cells reached 50% confluence, the transfection was carried out with the transfection mixture comprising 2.5 mL of serum-free medium, 6 μ L of target siRNA, and 3 μ L of Lipofectamine 2000 (11668-027, Invitrogen, USA). After incubation at room temperature for 20 min, the cells were transferred to wells, cultured at 37°C for 6-8 h under 5% CO₂. The complete medium was replaced, and the subsequent experiment was carried out after another 48 h of culture.

qRT-PCR

Quantitative real time polymerase chain reaction (qRT-PCR) was used to measure the mRNA levels of TCAB1, Wnt3a, β -catenin, caspase 9, Bcl-2, and Bax in the cells. RNA from the CSCs was extracted using Trizol reagent (15596-018, Beijing Solarbio Technology Co., Ltd., China), then dissolved with diethylpyrocarbonate (DEPC)-treated ultra-pure water (DEPC, SY0265, Beijing Biologics Technology Co., Ltd., China). An ultraviolet/visible spectrophotometer (SP-752, Shanghai Spectrum Instruments Co., Ltd., China) was used to measure the absorbance at 260 nm and 280 nm, then the concentration and quality of the total RNA were identified. Afterwards, the RNA was reverse-transcribed with a reverse transcription kit (RR037A, TaKaRa, China) using the two-step method. Reverse transcription system: 2 μ g RNA, RNase Free dH₂O up to 10 μ L, 2 μ L 5 \times PrimeScript Buffer (for Real Time), 0.5 μ L PrimeScript RT Enzyme Mix I, 0.5 μ L Oligo dT Primer (50 μ M), 0.5 μ L Random 6mers (100 μ M). The cDNA obtained was temporarily stored in a -80°C freezer. The performance of the qRT-PCR adopted the SYBR Green method. The reaction was operated according to the instructions of the kit (C11730-017, Invitrogen, USA). The primer sequences are shown in **Table 1**. Reaction conditions: pre-denaturation at 95°C for 30 s; degeneration at 95°C for 10 s; annealing at 60°C for 20 s, extension at 70°C for 10 s, for a total of 40 cycles. Reaction system: 1 μ L each forward and reverse primers, 12.5 μ L SYBR Green Mix, 1-4 μ L cDNA, ddH₂O up to 25 μ L. The qRT-PCR instrument used (ABI7900) was purchased from the ABI Company. Glyceral-

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dehyde-3-phosphate-dehydrogenase (GAPDH) served as an internal reference for the relative expression of all the genes, and the primers were synthesized using BGI Gene. The sequences are shown in **Table 1**. The melting curve evaluated the reliability of the PCR results. The cycle threshold (Ct) value (the inflection point of the amplification curve) was obtained, and $2^{-\Delta\Delta Ct}$ analyzed the relative expression of each target gene [23]. $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{internal reference gene}}$, $\Delta\Delta Ct = \Delta Ct_{\text{experimental group}} - \Delta Ct_{\text{control group}}$. The experiment was repeated 3 times and the average value was taken.

Western blot

The transfected cells were mixed with protein lysate (AR0107, Wuhan Boster Biological Technology, China), transferred to Eppendorf (EP) tubes, lysed on ice for 30 min, and centrifuged at 12000 rpm for 30 min at 4°C. The supernatant was collected and placed in an icebox. The protein concentration was determined using a bicinchoninic acid (BCA) kit (PC0020, Beijing Solarbio Technology Co., Ltd., China). 10% separating gel and 5% stacking gel were prepared using a sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) kit (P1200, Beijing Solarbio Technology Co., Ltd., China), then the protein was separated and transferred to a polyvinylidene difluoride (PVDF) membrane in a wet condition. Afterwards, the membrane was blocked for 1 h with 5% bovine serum albumin (BSA) at room temperature. After discarding the blocking agent, the membrane was placed in a plastic groove, and then 5% BSA was added to prepare the primary antibodies: rabbit anti-human TCAB1 polyclonal antibody (Abgent, USA), rabbit anti-human Wnt3a polyclonal antibody (1:1000, ab1678), rabbit anti-human β -catenin polyclonal antibody (1:500, ab8805), rabbit anti-human caspase 9 polyclonal antibody (1 μ g/mL, Ab25758), rabbit anti-human Bax polyclonal antibody (1:1000, ab53154), and rabbit anti-human Bcl-2 polyclonal antibody (1:1000, ab59348), which were purchased from the Abcam Company, UK. After incubation overnight at 4°C, the membrane was rinsed 3 times with Tris-Buffered Saline Tween-20 (TBST) for 10 min each time. Another 4-6 h incubation was carried out along with horseradish peroxidase-labeled goat anti-rabbit IgG (1:2000, ab6721, Abcam, UK) at 4°C, and the membrane was

washed 3 times with TBST for 15 min each time. The enhanced chemiluminescence (ECL) reagent A and reagent B (AR1111, Wuhan Boster Biology, China) were mixed evenly at a ratio of 1:1, then dropped onto a PVDF membrane for development. The optical density (OD) value of protein band was measured. The ratio of the gray values of the target band to the internal reference band indicated the relative expression of the target protein.

MTT assay

Transfected CSCs (20 μ L, 5×10^7 cells/mL) reaching 80% growth density were inoculated in a 96-well plate. MTT (M1025, Beijing Solarbio Technology Co., Ltd., China) solution (20 μ L, 5 mg/mL) was put into each well at the 24th, 48th, and 72nd hours of culture, and the supernatant and medium were discarded after another 4 h of culture. Dimethyl sulfoxide (DMSO, 150 μ L) (D8371, Beijing Solarbio Technology Co., Ltd., China) was added to each well and mixed for 10 min. The absorbance value (A) at the 490 nm wavelength was read using a microplate reader (Elx800, BioTek Instruments, USA) to determine the cell viability and to plot the cell growth curve.

Transwell

The apical chamber of the Transwell insert was paved with Matrigel (356234, Beijing Solarbio Technology Co., Ltd., China). Matrigel (50 μ L) diluted with serum-free RPMI 1640 medium (SNM550, Beijing Biologics Technology Co., Ltd., China) containing 0.2% BSA (1:5) was added to the apical chamber. The next day, 100 μ L of serum-free RPMI 1640 medium containing 0.2% BSA and 600 μ L of RPMI 1640 medium containing 10% FBS were added to the apical and basolateral chambers, respectively. The cells in each group were washed twice with PBS after routine digestion, 3 min each time. Afterwards, the cells were resuspended to a density of 1×10^5 cells/mL in an RPMI 1640 medium, then 200 μ L of cell suspension was added into the apical chamber. After routine culture for 24 h, the insert was taken out, and the excess cells in the apical chamber were wiped off with cotton swabs. The cells were then fixed for 15 min with 4% paraformaldehyde, stained for 15 min with 0.5% crystal violet solution prepared in methanol. After PBS

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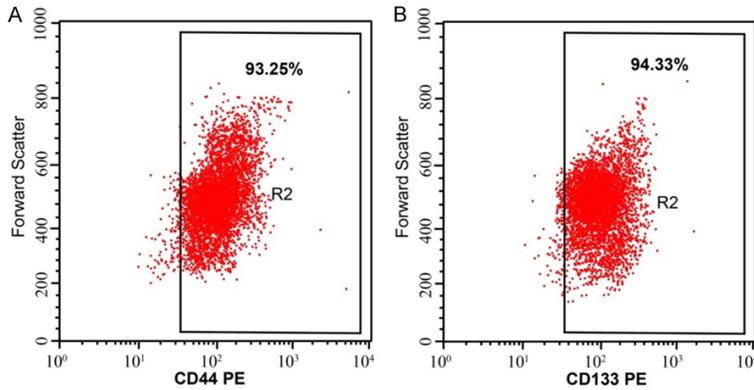


Figure 1. Flow cytometry separation results. A. The expression of CD44 in the OSCC cells after MACS; B. The expression of CD133 in the OSCC cells after MACS; MACS: magnetic-activated cell sorting; OSCC: oral squamous cell carcinoma.

washing, an inverted microscope (XDS-800D, Shanghai Caikon Optical Instruments Co., Ltd., China) was employed to measure the cell invasion in 5 randomly selected fields. Each group was provided with 3 repeating wells, and the average value was obtained after 3 repeated tests.

Flow cytometry

Measurement of the cell cycle: The concentration of transfected cells in each group was adjusted to 1×10^5 cells/mL after digestion with 0.25% trypsin. The cells were fixed with pre-cooled (-20°C) 75% ethanol, and allowed to stand overnight at 4°C . The next day, 1 mL of cells was centrifuged at 1500 r/min for 5 min, and the supernatant was discarded after the PBS washing, 1 mL of propidium iodide (PI, 50 mg/mL) (CA 1020, Beijing Solarbio Technology Co., Ltd., China) containing RNAase was added to the cell suspension, and the cell cycle was measured using flow cytometry (FACSCalibur, BD, USA) at the 575 nm excitation wavelength.

Quantification of the cell apoptosis: The concentration of transfected cells in each group was adjusted to 1×10^5 cells/mL after the digestion with trypsin. The cells were fixed with pre-cooled (-20°C) 75% ethanol, and allowed to stand overnight at 4°C . The next day, 1 mL of cells were centrifuged at 1500 r/min for 5 min, washed with PBS, resuspended with 200 μL of binding buffer, then mixed with 5 μL of Annexin V-fluorescein isothiocyanate (FITC) (CA1020, Beijing Solarbio Technology Co., Ltd., China)

and 10 μL of PI. The mixture was placed at room temperature for 15 min in the dark and mixed with 300 μL of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (HEPES) buffer. The cell apoptosis was measured using flow cytometry at the excitation wavelengths of 488 nm and 530 nm for the Annexin V-FITC and greater than 575 nm for the PI.

Statistical methods

All data were processed using SPSS 21.0 (SPSS, Chicago, Illinois, USA). The measurement data were expressed as the mean \pm standard deviation. Comparisons between two groups were done using independent samples t tests, and comparisons between multiple groups were done using one-way analyses of variance (ANOVA) and Bonferroni post hoc tests. The counting data were expressed as a percentage (%), and the comparisons were done using chi-squared tests. Values of $P < 0.05$ were considered statistically significant.

Results

Quantification of the expressions of the CSC markers in the OSCC cells using flow cytometry

Flow cytometry was employed to determine the expressions of CD44 and CD133 in the OSCC cells after MACS, and it turned out that the purity of the CD44⁺ and CD133⁺ cells were 93.25% and 94.33%, respectively (**Figure 1**), indicating that the majority of the sorted OSCC-CSCs were available for subsequent experiments.

Outcomes of qRT-PCR and Western blot

Compared with the blank group and the NC group, the expression of TCAB1 decreased significantly in the si-TCAB1 group and the combination group (all $P < 0.05$), but the expressions of Wnt3a, β -catenin, and Bcl-2 decreased in the si-TCAB1 group, the pathway inhibitor group, and the combination group, and the expressions of caspase 9 and Bax increased (all $P < 0.05$). There was no significant difference

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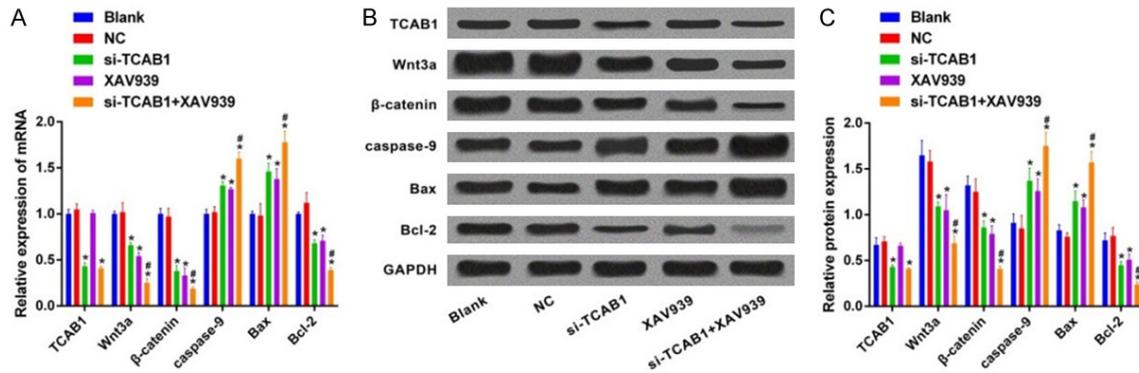


Figure 2. The mRNA and protein expressions of TCAB1, Wnt3a, β -catenin, caspase 9, Bax, and BCL 2 in each transfected cell group. A. Comparison of each group's mRNA expression of the related factors; B. The protein expression of the related factors in each group; C. The protein expression quantification of the related factors in each group; TCAB1: telomerase Cajal body protein 1; NC: negative control; Bax: BCL2-Associated X; Bcl-2: B-cell lymphoma-2. Compared with the blank group and the NC group, $^*P<0.05$; Compared with the siRNA-TCAB1 group, $^{\#}P<0.05$.

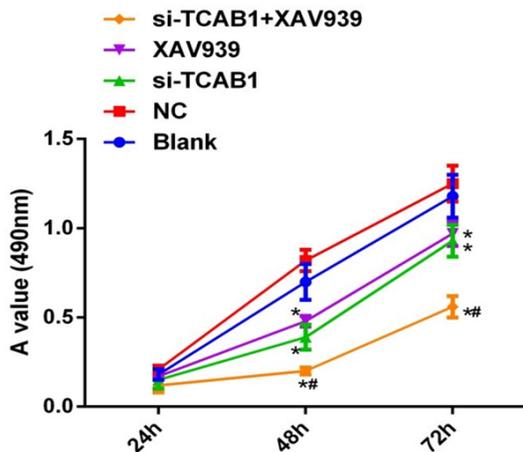


Figure 3. Proliferation of the cells in each group after transfection. TCAB1: telomerase Cajal body protein 1; NC: negative control. Compared with the blank group and the NC group, $^*P<0.05$; Compared with the siRNA-TCAB1 group, $^{\#}P<0.05$.

between the blank group and the NC group (all $P>0.05$). Compared with the si-TCAB1 group, the expression of TCAB1 increased in the pathway inhibitor group ($P<0.05$), but the other indexes showed no statistical differences (all $P>0.05$), and the expressions of Wnt3a, β -catenin, and Bcl-2 decreased, but caspase 9, and Bax increased in the combination group (all $P<0.05$). It was suggested that knocking down TCAB1 expression or inhibiting the Wnt/ β -catenin signaling pathway hindered the proliferation and promoted the apoptosis of OSCC-CSCs, as shown in **Figure 2**.

Cell proliferation

An MTT assay was adopted to determine the proliferation of the cells in each group, and the outcomes are shown in **Figure 3**. The proliferation in each group was not significantly different at the 24th hour of culture (all $P>0.05$). There was no significant difference between the blank group and the NC group at each time point (all $P>0.05$). At the 48th and 72nd hour of culture, the proliferation of the CSCs in the si-TCAB1 group, the pathway inhibitor group, and the combination group was slowed down compared with the blank group and the NC group, and the retardation in the combination group was more significant (all $P<0.05$). There was no significant difference in the si-TCAB1 group or the pathway inhibitor group at each time point (all $P>0.05$). The results showed that knocking down TCAB1 or inhibiting the activation of the Wnt/ β -catenin signaling pathway inhibited the proliferation of CSCs.

Cell invasion

The outcomes of Transwell are displayed in **Figure 4**. No significant differences showed between the blank group and the NC group ($P>0.05$). Compared with the Blank group, the cell invasion in the si-TCAB1 group, the pathway inhibitor group, and the combination group was reduced, and the reduction in the combination group was more significant ($P<0.05$), but the difference between the si-TCAB1 group and the pathway inhibitor group was not significant (all

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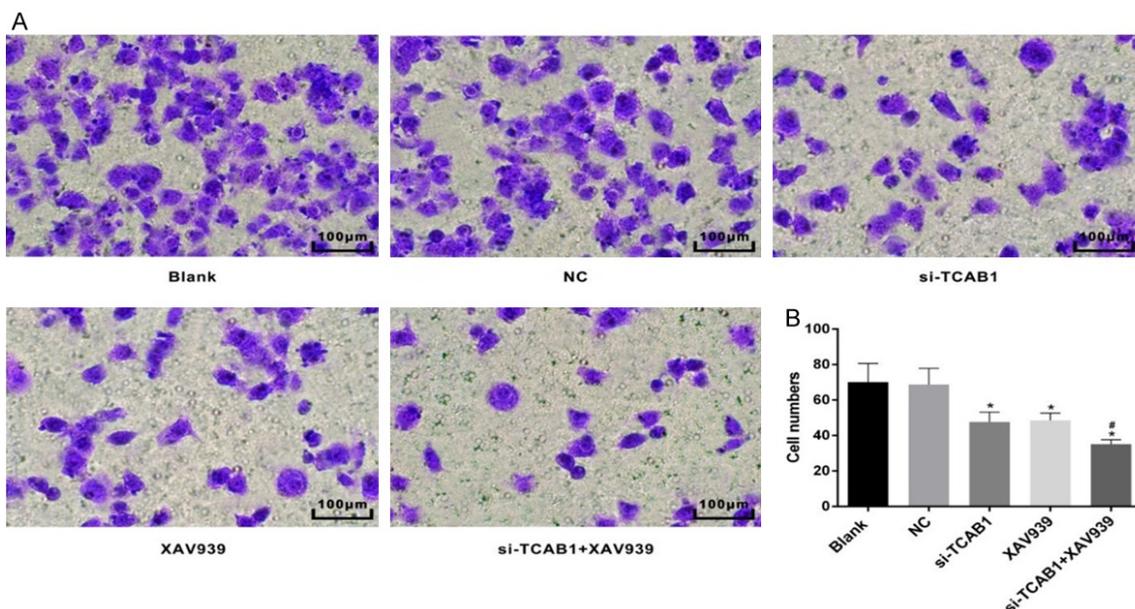


Figure 4. The effect of the migration and invasion of the cancer stem cells in each group after transfection. A. Determination of the cell invasion in each group using Transwell; B. Statistical histogram of the cell invasion in each group; TCAB1: telomerase Cajal body protein 1; NC: negative control. Compared with the blank group and the NC group, * $P < 0.05$; Compared with siRNA-TCAB1 group, # $P < 0.05$.

$P > 0.05$). These outcomes indicated that knocking down TCAB1 or inhibiting the Wnt/ β -catenin signaling pathway effectively decreased the invasion of the OSCC-CSCs.

Cell-cycle distribution

The outcomes of PI staining are shown in **Figure 5**. Compared with the blank group and the NC group, the changes of the CSC cycle in the si-TCAB1 group, the pathway inhibitor group, and the combination group were mainly manifested in the G0/G1-phase extension and the s-phase shortening (all $P < 0.05$), but no significant difference showed between the si-TCAB1 group and the pathway inhibitor group (all $P > 0.05$). Compared with the si-TCAB1 group, the G0/G1-phase was extended and the s-phase was shortened in the combination group (all $P < 0.05$). It was turned out that knocking down the TCAB1 or inhibiting the Wnt/ β -catenin signaling pathway inhibited the proliferation of the CSCs.

Cell apoptosis

The results of the flow cytometry to measure the apoptosis in each group are shown in **Figure 6**. There was no significant difference in the apoptosis between the blank group and the NC group ($P > 0.05$). Compared with the blank group

and the NC group, the apoptosis of the CSCs in the si-TCAB1 group, the pathway inhibitor group, and the combination group increased, and the increase in the combination group was more significant (all $P < 0.05$). The results showed that the TCAB1 knock-down or inhibition of the Wnt/ β -catenin signaling pathway accelerates the apoptosis of the CSCs.

Discussion

OSCC is a common epithelial malignant solid tumor with the highest prevalence among oral and maxillofacial malignant tumors [1, 24-26]. Although the survival of most patients has been prolonged after radiotherapy and chemotherapy, the recurrence and metastasis rates still remain high [27]. Squamous cell carcinoma has heterogeneity, and its cellular structure and marker expression are similar to those of human normal oral epithelia; moreover, basal-like cells located at the edge of cancer nests are also found to have stem cell properties [28]. There are large numbers of CSCs in lesions after chemotherapy, indicating that traditional treatments fail to eradicate CSCs. Therefore, CSCs play a vital role in identifying the development, progression, and drug resistance of tumors, as well as in eliminating CSCs and cur-

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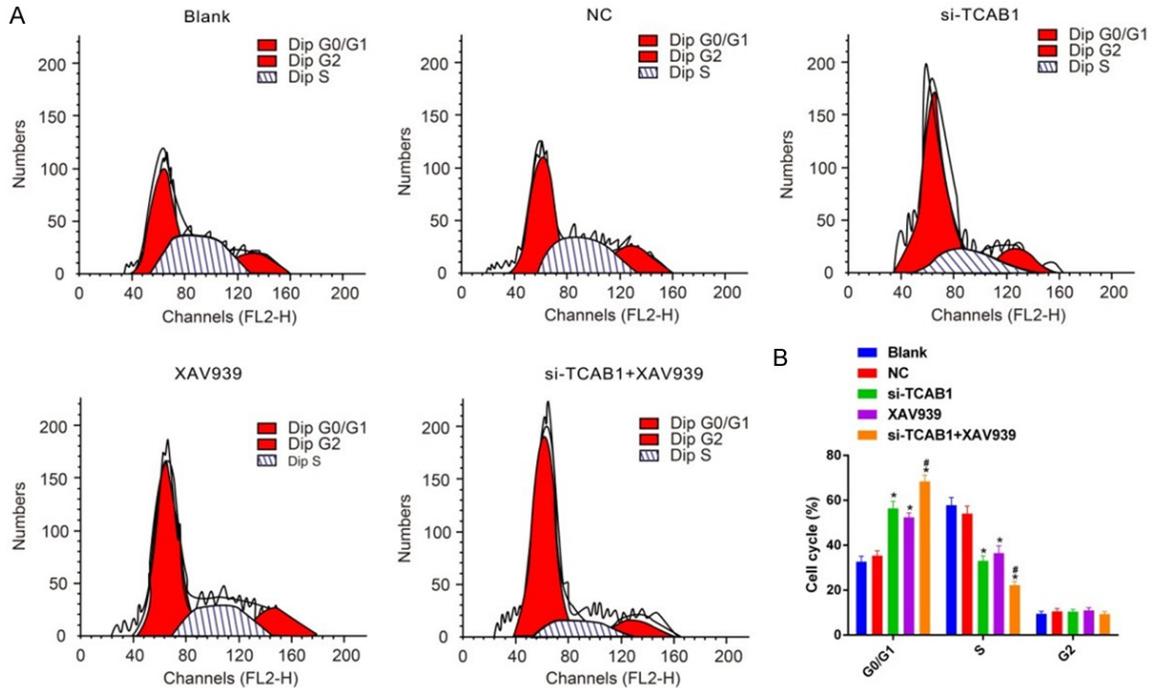


Figure 5. Comparison of the cell cycle in each group after transfection. A. Flow cytometry of each group after the cell transfer; B. Changes in the cell cycle in each group after transfection. Compared with the blank group and the NC group, * $P<0.05$; Compared with siRNA-TCAB1 group, # $P<0.05$.

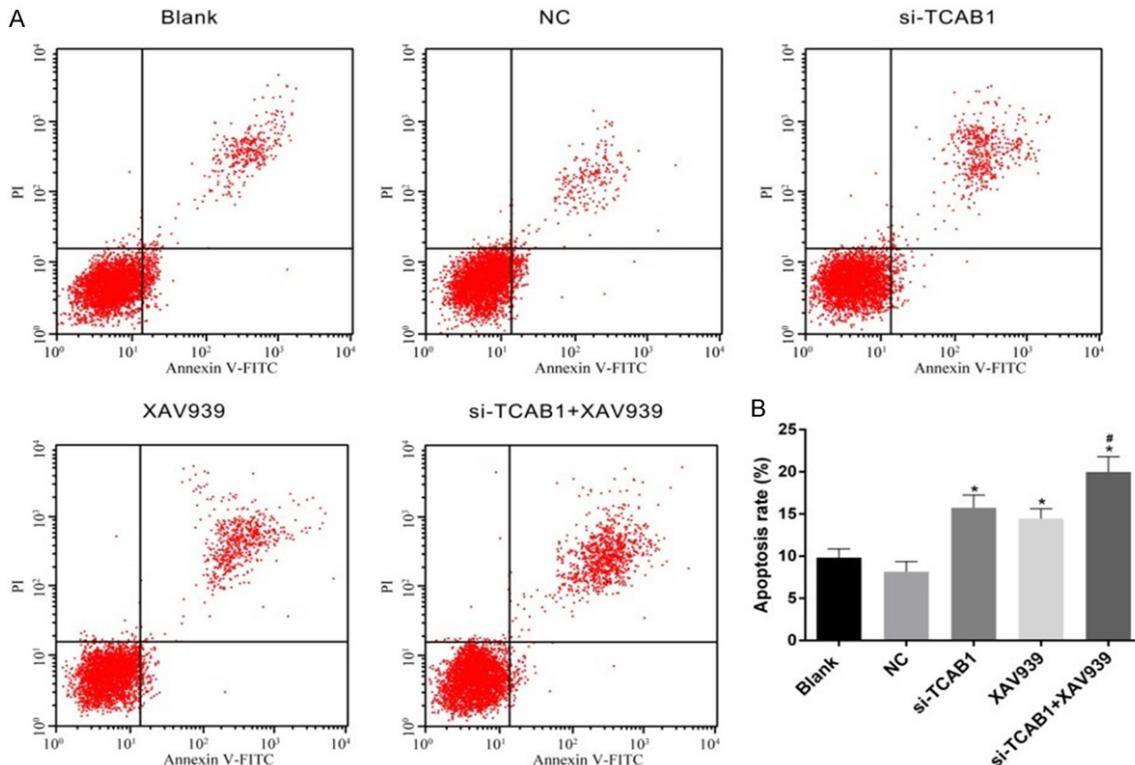


Figure 6. Comparison of the apoptosis in each group after the transfection. A. The apoptosis in each group after transfection; B. The apoptosis rate of each group after the cell transfection. Compared with the blank group and the NC group, * $P<0.05$; Compared with the siRNA-TCAB1 group, # $P<0.05$.

ing tumors [29]. In this study, it was speculated that interfering with gene expression might regulate CSC properties, and we eventually confirmed that interfering with TCAB1 promoted apoptosis and inhibited the proliferation and invasion of OSCC-CSCs by regulating the Wnt/ β -catenin pathway.

TCAB1, the core protein of telomerase, is the key to maintaining the length and function of telomerase. The inhibition of TCAB1 expression shortens telomeres and accelerates cancer cell death [30]. Wang et al. stated that TCAB1 expression was significantly elevated in NPC, and its deletion could lead to cell cycle arrest and the accelerated apoptosis of NPC cells [9]. Yuan et al. confirmed that TCAB1 knock-out significantly inhibited the proliferation of NSCLC cells [10]. Another study also demonstrated that the expression of TCAB1 was up-regulated in head-and-neck cancer, and the suppression of TCAB1 inhibited tumor formation in nude mice *in vitro* [11]. Based on previous studies, this study further explored the effects of TCAB1 knock-down on OSCC-CSCs.

The Wnt/ β -catenin signaling pathway has been shown to play a regulatory role in cell proliferation, apoptosis, and biological development, and its over-activation is closely associated with tumor progression [31-34]. In the study by Prgomet, the expression of Wnt5a was significantly enhanced in OSCC, which accelerated the proliferation and invasion of cancer cells by activating non-canonical Wnt signals [35]. In addition, the expression of long non-coding RNA urothelial carcinoma-associated 1 (lncRNA UCA1) was reported to be significantly enhanced in OSCC, and its regulation of the proliferation and migration of OSCC cells was mainly realized by activating the Wnt/ β -catenin signaling pathway [36]. Yu et al. also revealed that inhibiting the activation of the Wnt/ β -catenin pathway effectively hindered the proliferation of OSCC cells [37]. In this study, we found that the expression of the Wnt/ β -catenin pathway-related factors was significantly reduced after TCAB1 suppression, indicating the inhibited activation of the pathway. A previous study demonstrated that TCAB1 regulated the MD-M2-p53 signaling pathway by affecting the ubiquitination of murine double minute 2 (MDM2) and p53 before proteasome degradation [18]. However, the specific mechanism by

which TCAB1 regulates Wnt/ β -catenin has not been explored in depth, and it will be the focus of our follow-up research. qRT-PCR and Western blot illustrated that knocking down TCAB1 or inhibiting the Wnt/ β -catenin pathway suppressed the expression of the apoptosis inhibitor Bcl-2 in OSCC-CSCs but elevated the expression of the apoptosis promoter Bax and the apoptosis effector caspase-9, indicating that the TCAB1 knockdown inhibited the Wnt/ β -catenin signaling pathway and further accelerated the apoptosis of CSCs. The cell proliferation, invasion, cycle, and apoptosis were measured using MTT assays, Transwell, PI staining, and flow cytometry, respectively. The results showed that TCAB1 knock-down inhibited the Wnt/ β -catenin signaling pathway and hindered the proliferation and invasion of OSCC-CSCs, resulting in the remarkable G0/G1 arrest and an increased rate of cell apoptosis.

In conclusion, TCAB1 knockdown inhibits the Wnt/ β -catenin signaling pathway and hinders the proliferation and invasion of, and accelerates the apoptosis of OSCC-CSCs. This study explored the effects of TCAB1 on OSCC-CSCs and its potential specific mechanism, which is of great significance for clarifying the pathogenesis of OSCC and adopting targeted treatment regimens. However, there are still several limitations, for example, the effects of TCAB1 on the cell death mode, the TCAB1 regulated changes in other proteins, and the specific ways TCAB1 affects the Wnt/ β -catenin signaling pathway, all of which have not been investigated, but they will be addressed in future experiments.

Disclosure of conflict of interest

None.

Address correspondence to: Changxue Li, Department of Stomatology, The First Affiliated Hospital, School of Medicine, Shihezi University, No. 107 North Second Road, Shihezi 832000, Xinjiang Uygur Autonomous Region, China. Tel: +86-0993-285-8573; E-mail: lichangxueshz1h@163.com

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