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Nicotine may promote tongue squamous cell carcinoma progression by activating the Wnt/β-catenin and Wnt/PCP signaling pathways

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Abstract. To investigate the effects and the possible underlying mechanisms of nicotine stimulation on tongue squamous cell carcinoma (TSCC) progression, a TSCC cell line Cal27 and 34 samples of paraffin-embedded TSCC were examined. Immunofluorescence, western blot analysis, and TOP/FOP flash, CCK-8, wound healing and Transwell invasion assays were used to evaluate Cal27 in response to nicotine stimulation. We also investigated expression levels of related proteins of Wnt/β-catenin and Wnt/PCP pathways in paraffin-embedded TSCC samples with or without a history of smoking by immunohistochemistry. Nicotine stimulation can promote proliferation, migration, and invasion of TSCC cells *in vitro*, downregulate E-cadherin, and activate the Wnt/β-catenin and Wnt/PCP pathways, which could be antagonized by the α7 nicotine acetylcholine receptor (α7 nAChR) inhibitor α-BTX. Moreover, the expression levels of β-catenin, Wnt5a and Ror2 were higher in TSCC patients with a history of smoking than those without a history of smoking. Our results suggest nicotine may promote tongue squamous carcinoma cells progression by activating the Wnt/β-catenin and Wnt/PCP signaling pathways and may play a significant role in the progression and metastasis of smoking-related TSCC.

Introduction

Tongue squamous cell carcinoma is one of the most common cancers in the neck and head (1), which often results in short survival and poor prognosis, even after surgery and chemotherapy. Previous studies have revealed that TSCC is associated with a high incidence of tobacco abuse. However, the mechanism for how tobacco promotes the progression of TSCC is still unclear.

Nicotine, the addictive component in cigarettes, has been shown to promote tumor cell proliferation and metastasis by promoting cell-cycle progression, epithelial-to-mesenchymal transition (EMT), migration, invasion, angiogenesis, and anti-apoptosis through several signaling pathways (2). Nicotine is thought to promote tumor progression by binding to nicotinic acetylcholine receptors and stimulate multiple cancer-promoting signaling cascades, such as VEGFR, JAK/STAT, PI3K/Akt, and Ras/Raf/MEK/ERK signaling pathways (3-5). Specifically, it is been shown that nicotine stimulation can induce proliferation, angiogenesis and EMT in non-small cell lung cancer cells and esophageal squamous cell cancer (6-8). Nicotine can even promote EMT via Wnt/β-catenin signaling in normal human airway epithelial cells and human alveolar interstitial fibroblasts (9,10). In these recent studies, nicotine has been found to contribute to the progression and metastasis of tumor cells or normal cells by activation of several signaling pathways.

So far several studies have investigated the physiologic nicotine concentration of tobacco consumers and find that nicotine is present in the plasma and saliva at levels ranging from 0.03 to 0.5 μM (11), and 0.6 μM to 10 mM (12), respectively. Epithelial cells act as a mechanical barrier to reduce a portion of the nicotine and make it impossible to reach 10 mM in the tongue tissue. In many experiments about nicotine stimulation to lung tissue, various concentrations of nicotine have been used (6,7,9,10,13,14). They chose 1, 5, 0.08 or 6 μM to conduct subsequent functional experiments. Combining the physiological plasma and saliva nicotine concentration and above previous studies about the effects of nicotine to lung tissue, we carefully selected to use nicotine from 0.1 to 10 μM in our study.

The effects of nicotine stimulation on TSCC are unknown, and the underlying mechanism is not clear. According to previous studies, the activation of canonical Wnt/β-catenin signaling promotes invasion, proliferation and anti-apoptosis in TSCC cells (15-17). Thus we have attempted to explore the

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possible connection between nicotine stimulation and activation of the Wnt/β-catenin pathway in TSCC. In addition, the PI3 K/AKT pathway can be activated after nicotine stimulation, and the Wnt/PCP pathway can crosstalk with the PI3k/AKT pathway and Wnt/β-catenin pathway (18). Therefore, we aimed to determine whether Wnt/PCP pathway is also affected by nicotine stimulation in TSCC.

Materials and methods

Antibodies and reagents. Rabbit monoclonal β-catenin, Ror2 (tyrosine-protein kinase transmembrane receptor Ror2) and c-Myc antibodies were purchased from Abcam Inc. (Cambridge, MA, USA; cat. nos. ab32572, ab92379 and ab32072). Rabbit monoclonal phospho-Akt (Ser473) and phospho-GSK-3β antibodies were from Cell Signaling Technologies (Beverly, MA, USA; cat. nos. 4060 and 5558). Rabbit polyclonal E-cadherin, Wnt5a and c-jun antibodies were obtained from Wanlei Bio (Shenyang, China; cat. nos. wl01482, wl0198 and wl0219a). The α7 nAChR antibody was from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA; cat. no. sc-5544). Goat monoclonal HRP-conjugated antibody against GAPDH and goat anti-rabbit HRP-conjugated secondary antibodies were obtained from Dingguo Biological Technology (Beijing, China; cat. no. SH-0031). Nicotine was purchased from Sigma-Aldrich (St. Louis, MO, USA). α-Bungarotoxin (α-BTX), a specific antagonist of α7 nAChR, was purchased from Tocris (Bristol, UK).

Tissue specimens. Tongue squamous cell carcinoma tissue specimens were collected at Stomatology Hospital of Shandong University (Jinan, China). Upon recruitment, informed consent was obtained from each subject. This study was approved by the Institutional Research Ethics Committee of School of Stomatology, Shandong University.

Cell culture. The tongue cancer cell line Cal27 was obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cal27 has been identified by the measurement of short tandem repeats. The cells were passaged approximately 6 times in our laboratory during a period of fewer than 6 months after reidentification. Cal27 was cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) containing 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cal27 cells were cultured at 37˚C in a humidified atmosphere of 95% air and 5% CO_2.

Cell proliferation assay. Cells were cultured in 96-well tissue culture plates ($3x10^3$ cells/well) with 10% FBS for 24 h. Then, the cells were exposed to 0.1 μM nicotine for 24, 48, 72, 96 and 120 h. Cell proliferation was measured by a CCK-8 assay (19). Briefly, 10 μl CCK-8 solution was added to each well, and the plates were incubated for additional 2 h. The absorbance was measured using a spectrometer at a wave length of 450 nm. Cell proliferation was also assessed using the clonogenic formation assay (20). Briefly, 1,000 Cal27 cells were seeded per well of a 6-well plate. Cells were treated with the indicated concentration of nicotine with or without α-BTX for one week to determine the impact of nicotine on proliferation. After one week, colonies were paraformaldehyde-fixed, and the number of colonies was determined after crystal violet staining as previously described (20).

Wound healing assay. Cells ($5x10^5$ cells/well) were seeded in 6-well plates and allowed to attach to 80% confluence. Cell monolayers were wounded by scratching with 200-μl pipette tips before being washed twice with phosphate-bufferred saline (PBS) to remove floating cells. Cells in the each well were subsequently exposed to serum-free DMEM with or without nicotine for up to 24 h. Cells were photographed at x100 magnification under a phase-contrast microscope at each time-point. The healing area at different times was measured using Image-Pro Plus 6.0 software.

Transwell invasion assay. Cal27 ($2x10^4$ cells/0.4 ml) cells were seeded in the upper chamber of the Transwell inserts (8-μm pore size) pre-coated with Matrigel (both from Corning, Corning, NY, USA) and exposed to FBS-free medium with or without 10 μM nicotine. Medium containing 10% FBS was placed in the lower chamber, and cells for each treatment were incubated for 24 h at 37˚C in a humidified atmosphere with 95% air and 5% CO_2. Then, the non-invasive cells in the upper chamber were removed with a cotton swab, and the invaded cells were fixed with 4% formaldehyde for 15 min and then stained with 0.1% crystal violet in 0.01 M PBS for 15 min after being washed with PBS. The number of cells that penetrated the membrane was counted, and images were captured under a light microscope at a magnification of x200, as previously described (21).

TOP/FOP flash assay. TOP/FOP flash assay is a luciferase reporter assay which is used to assess the activity of β-catenin and T-cell factor (TCF) signaling. The cells ($3x10^4$ cells/well in 24-well plates) were transfected with 0.1 g of TOPflash or FOPflash (Upstate Biotechnology, Lake Placid, NY, USA) and 5 ng of pRL-SV40 (Promega, Madison, WI, USA) using Lipofectamine 2000 reagent (Invitrogen). After 24 h, the cells were switched to normal complete medium with or without 10 μm nicotine for another 24 h. Then, the cells were lysed, and the luciferase activity was determined according to the manufacturer's recommendations. The luciferase activity of each sample was normalized with its respective *Renilla* luciferase activity.

Western blot. Cells were harvested and lysed, and protein concentration was determined by the BCA assay. Equal quantities of protein were separated on 10% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking in 5% fat-free dry milk in Tween-20 Tris-buffered saline (TBST) for one hour, the membranes were probed with antibody against β-catenin (1:5,000), Ror2 (1:2,000), c-Myc (1:1,000), E-cadherin (1:3,000), Wnt5a (1:1,000), c-jun (1:500), p-Akt (1:1,000), p-GSK3β (1:1,000), GAPDH (1:20,000) and α7 nAChR (1:1,000), washed in TBST and then incubated with HRP-conjugated secondary antibody (1:20,000). Finally, protein bands were detected using the Immobilon western chemiluminescent HRP substrate kit.

Immunofluorescence and Immunohistochemical staining. Immunohistochemistry (IHC) analysis was performed to

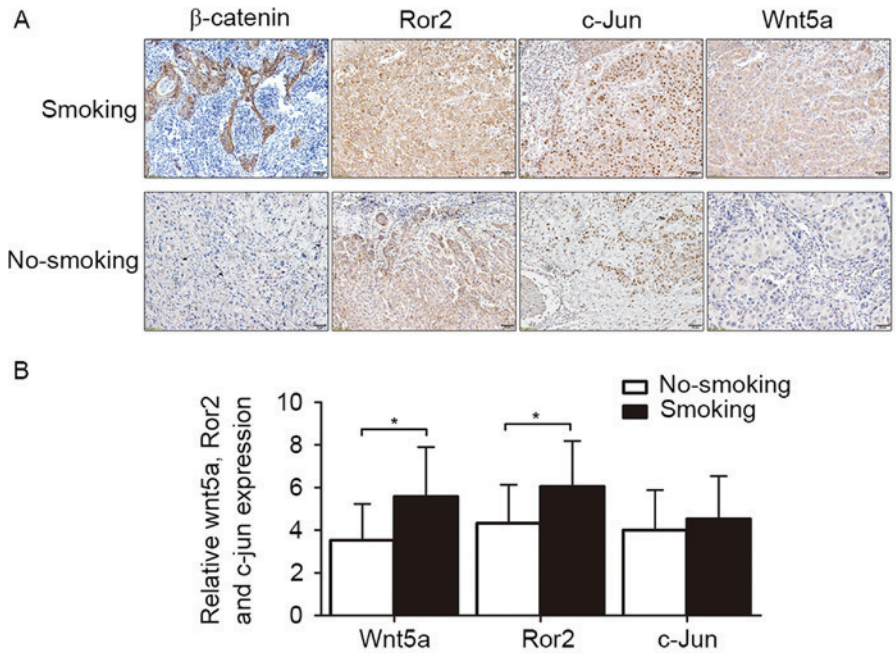

Figure 1. The expression levels of β-catenin, Wnt5a and Ror2 in human TSCC with smoking history were higher than those without a history of smoking. (A) Representative IHC stained sections showed different expression patterns of β-catenin, Wnt5a, c-jun and Ror2 in TSCC with or without smoking history (original magnification, x200). (B) The quantitative results of Wnt5a, c-jun and Ror2 expression in TSCC with or without smoking history. *P<0.05.

investigate the expression of β-catenin and Ror2 in different samples of human tongue cancer. Briefly, the sections were deparaffinized in xylene, hydrated through a graded alcohol series, and washed with PBS. Antigen retrieval was performed by treatment with 10 mM sodium citrate buffer (Zhongshan, Beijing, China) in a pressure cooker for 5 min. The activity of endogenous tissue peroxidase was blocked with 3% H_2O_2 (Zhongshan) for 30 min. After pretreatment with normal goat serum (Zhongshan) for 30 min to block nonspecific binding, the sections were incubated with β-catenin (1:500) and Ror2 (1:250) at 4˚C overnight. Sections treated with PBS instead of the primary antibody were used as negative controls. The sections were incubated with HRP-conjugated goat-anti-rabbit secondary antibody for 30 min, followed by reaction with diaminobenzidine, and counterstaining with Mayer hematoxylin. For evaluation of Ror2 (cytoplasmic and membranous staining), c-jun and Wnt5a (cytoplasmic and nuclear staining), we used a semiquantitative approach based on staining intensity (SI) and percentage of positive cells (PP), to create the immunoractive score (IRS) as follows: IRS=SIxPP, for each sample, as previously described (22). Intensity was scored as follows: 0, no staining; 1, weakly positive; 2, moderately positive; and 3, strongly positive. The scoring of the staining pattern was based on the percentage of positive tumor cells: 0, 0-5%; 1, 6-25%; 2, 26-50%; and 3, 51-100%). The IRS score ranged from 0 to 9. To evaluation of β-catenin expression, we used a method described by Maruyama *et al* (23). Normal expression was defined as positive membrane staining seen in >70% cells, otherwise, it was deemed as a deletion of membrane expression. Positive cytoplasmic and nuclear expression was defined when staining was observed in >10% cells. Deletion of membrane expression and positive cytoplasmic and nuclear expression were proposed as defined abnormal expression.

For immunofluorescence assays, cells were seeded on glass coverslips in a 6-well plate for 24-48 h with or without nicotine stimulation. Then, cells were fixed with paraformaldehyde for 15 min at room temperature and washed with PBS. After blocking with normal goat serum, cells were incubated with associated antibody (β-catenin, 1:200) overnight at 4˚C. Then, slides were washed and incubated with FITC-conjugated goat anti-rabbit IgG for 1 h at room temperature. Slides were washed with PBS again before being stained with DAPI and examined with a fluorescence microscope.

Statistical analysis. All statistical analyses were performed using SPSS version 19.0 (SPSS, Chicago, IL, USA). Student's *t*-test or analysis of variance was used to compare group distributions. All results were expressed as mean ± standard deviation (SD). A value of P<0.05 was considered statistically significant.

Results

The expression levels of β-catenin, Wnt5a, and Ror2 are associated with smoking history in TSCC patients. A previous study has reported that cigarette smoke extract can activate Wnt/β-catenin pathway *in vitro* (21) and promote the expression of Wnt5a *in vivo* (24). To investigate relationship between smoking history and the expression levels of β-catenin, Wnt5a, c-jun and Ror2 in human TSCC, IHC was performed to determine expression levels of β-catenin, Wnt5a, c-jun and Ror2 in paraffin-embedded TSCC tissues (smoking patients, n=18; non-smoking patients, n=15). IHC staining revealed that Wnt5a (IRS=5.58±2.32) and Ror2 (IRS=6.05±2.13) were significantly higher in tumor tissues of smoking patients than those (IRS=3.53±2.17) and (IRS=4.33±2.02) of non-smoking patients (P=0.013 and P=0.019) (Fig. 1). In smoking group,

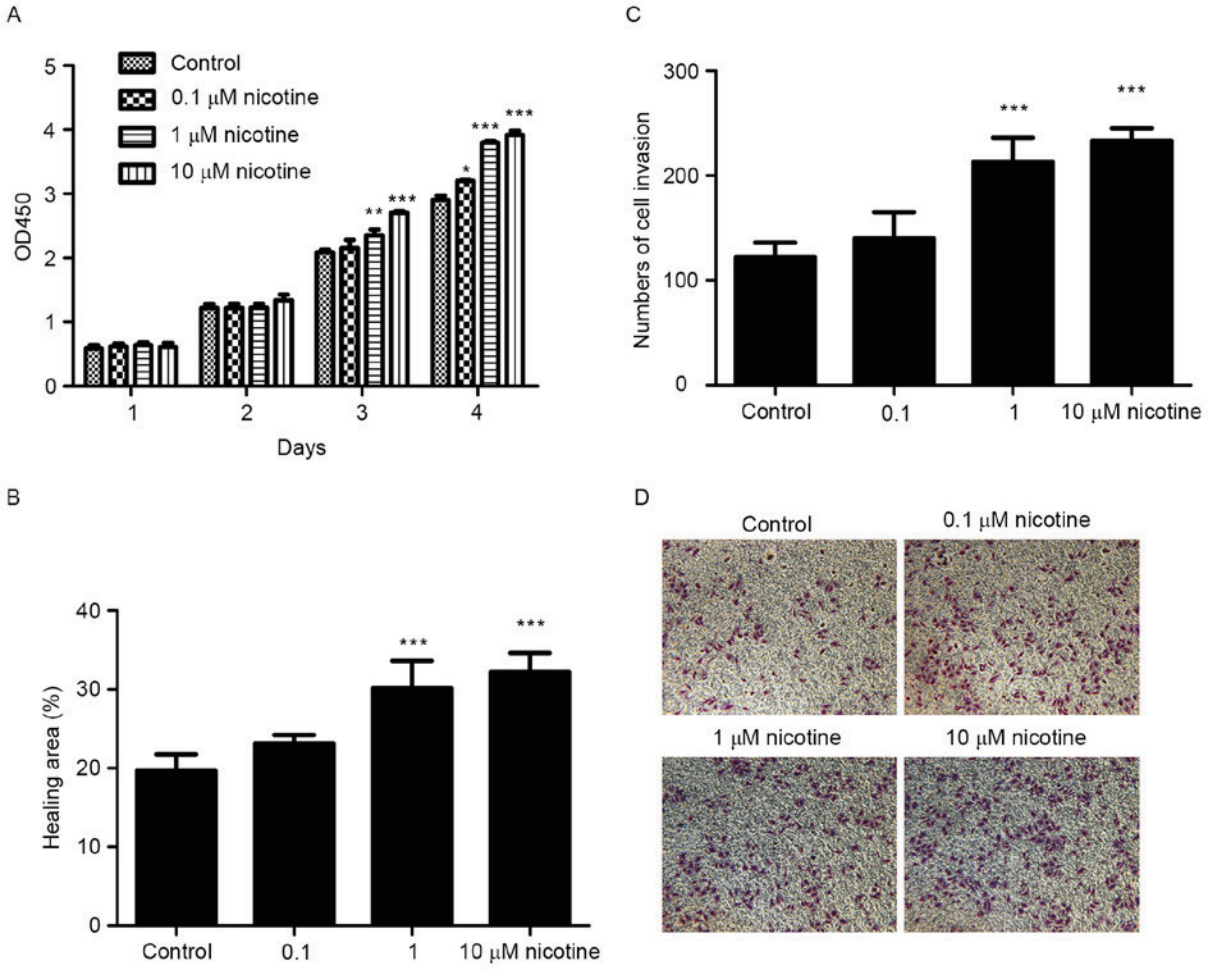

Figure 2. Nicotine promoted the proliferation, migration and invasion of TSCC cells. (A) Cell proliferation was measured by a CCK-8 cell proliferation assay at the indicated time-point and nicotine concentration. Data are presented as the mean ± SD. (B) Cell migration was measured by wound healing assay at indicated concentration of nicotine stimulation for 12 h. Student's *t*-test was used to compare the speed of wound healing between the nicotine stimulation group and the control group (original magnification, x100). (C and D) Images of the Transwell invasion of Cal27 cells after different treatments were analyzed using Image-Pro Plus 6.0 software. The number of cells invaded was counted and expressed as the mean ± SD (original magnification, x200). (*P<0.05, **P<0.01, ***P<0.001) .

89.47% (16/18) of patients had abnormal expression of β-catenin but only 53.33% (8/15) in non-smoking group (P=0.018). In addition, the expression of c-jun were not significantly associated with a smoking history (IRS=4.53±2.01 vs. IRS=4.00±1.89, P=0.442). This result suggests the elevated expression of β-catenin, Wnt5 and Ror2 are potentially connected to smoking history in TSCC patients. Based on the IHC results, we presume that Wnt/β-catenin pathway and Wnt/PCP pathway in TSCC cells may be overactivated by nicotine stimulation as well *in vitro.*

Nicotine stimulation promotes proliferation, migration and invasion of Cal27 cells. To investigate the effects of nicotine on proliferation, we treated cells with different concentrations of nicotine and measured the change of cell proliferation by CCK-8 assay. As expected, the proliferation of Cal27 cells was promoted by nicotine in a time and dose dependent manner (Fig. 2A). When incubated for 72 h, the number of cells in the 10 μM nicotine group was significantly greater than in that in the control group (2.71±0.03 vs. 2.08±0.09, P<0.001). In the wound healing assay, nicotine stimulation reduced the time of scratch healing (Fig. 2B). For example, at 12 h 32.2±2.43% of the wound was healed in the 10 μM nicotine group, but only 19.67±2.08% was healed in the control group in Cal27 cells (P<0.001). Similarly, cells' invasion ability after nicotine stimulation was improved as determined by Transwell invasion assays (Fig. 2C). These results suggest that nicotine can promote proliferation, migration and invasion of Cal27 cells *in vitro.*

Nicotine can activate Wnt/β-catenin and the Wnt/PCP pathways in Cal27. To test our hypothesis, cells were stimulated by nicotine *in vitro*, and expression levels of related proteins were determined by western blot analysis and immunofluorescence. We found that the expression levels of total β-catenin and nuclear β-catenin were increased in Cal27 after nicotine simulation (Fig. 3A and C), and western blot analysis detected that p-GSK3β was increased as well (Fig. 3A). The expression level of c-Myc, a downstream target gene of Wnt/β-catenin pathway, was increased in a dose-dependent manner upon nicotine stimulation (Fig. 3A). Additionally, we found that Wnt/β-catenin signaling activity was substantially higher in Cal27 cells treated with nicotine compared to the control group, as determined using the TCF-dependent TOPflash

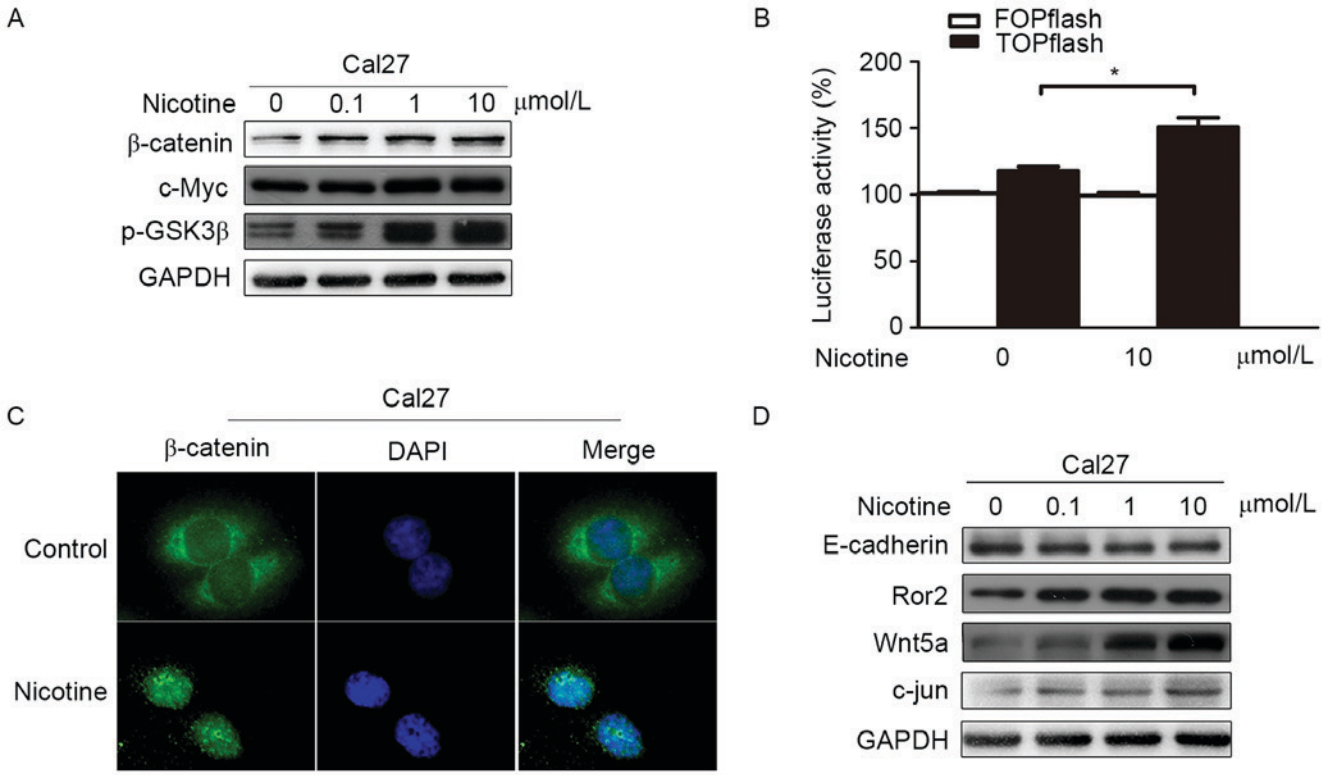

Figure 3. Nicotine induced the activation of the Wnt/β-catenin and Wnt/PCP pathways. (A) Total β-catenin expression and c-Myc expression of Cal27 were increased by nicotine stimulation for 48 h in a dose-dependent manner as determined by western blot analysis. Nicotine stimulation also promoted p-GSK3β expression in a dose-dependent manner in Cal27 cells. (B) After transfection with the TOPflash or control FOPflash reporter, luciferase values were normalized to β-galactosidase expression. Data are presented as the mean ± SD. (*P<0.05) (C) Expression and subcellular localization of β-catenin was examined with fluorescence microscopy. Translocation of β-catenin (green) from the cytoplasm to the nucleus was observed in Cal27 cells after 10 μM nicotine stimulation for 48 h. (D) Ror2, Wnt5a and c-jun expression in Cal27 cells was increased, and E-cadherin expression was decreased, by nicotine stimulation for 48 h in a dose-dependent manner as determined by western blot analysis.

reporter (Fig. 3B). Taken together, these results suggest that the Wnt/β-catenin pathway is activated by nicotine stimulation.

Western blot analysis also detected that levels of Ror2, Wnt5a and c-jun, a downstream effector of the Wnt/PCP pathway, were significantly increased in the nicotine stimulation condition in a dose-dependent manner (Fig. 3D). Therefore, in the presence of nicotine, the Wnt/PCP pathway is also activated in Cal27 cells *in vitro*.

Nicotine may promote progression of Cal27 cells by activating the Wnt/β-catenin and Wnt/PCP pathways through α7 nAChR. Our previous research indicated that Wnt/PCP pathway can facilitate migration and invasion of Cal27 cells (25). Previous studies showed that nicotine can promote proliferation and invasion in several cancers via the Wnt/β-catenin pathway (9,13). In this study, we examined if the activation of Wnt/β-catenin and Wnt/PCP pathways by nicotine involved α7 nAChR in Cal27 cells. We found that the upregulation of β-catenin, p-GSK-3β, Wnt5a, Ror2 and c-jun was reversed when co-treated with the α7 nAChR inhibitor α-BTX (Fig. 4A) in Cal27 cells. In addition, the results of colony formation, Transwell and migration assays revealed that the stimulating effects of nicotine on proliferation, invasion, and migration of Cal27 cells could be antagonized if co-treated with α-BTX. The number of colonies formed in the nicotine group was 318.66±29.67, while only 212.54±24.03 colonies formed in the control group (P=0.009) and the nicotine-induced proliferation was reversed when co-treated by α-BTX (224.32±28.15, P=0.016) (Fig. 4B). In the wound healing assay, at 12 h, 37.2±2.21% of the wound was healed in the nicotine group, while only 29.21±1.18% was healed in the α-BTX co-treated group in Cal27 cells (P=0.005) (Fig. 4C). Based on the results above, the change of biological characteristics of Cal27 cells by nicotine stimulation may be explained by the activation of the Wnt/β-catenin and Wnt/PCP pathways, a process that requires α7 nAChR.

Additionally, we have found that E-cadherin expression was decreased (Fig. 2D), which could explain the aggressive migration of Cal27 in the presence of nicotine.

Discussion

Tongue squamous cell carcinoma is one of the most common and malignant cancers affecting the oral cavity. After lymph node or distant metastasis, TSCC patients will have a poor prognosis even after systemic therapy (26). Therefore, it is critical to explore and understand the mechanism of TSCC development and metastasis, which may help us to interrupt tongue cancer progression.

In our study, we determined that nicotine stimulation could promote the proliferative and invasive abilities of TSCC cells in accordance with a previous study (27). These findings suggest that TSCC patients with a history of smoking should receive specific care and should be encouraged to quit smoking.

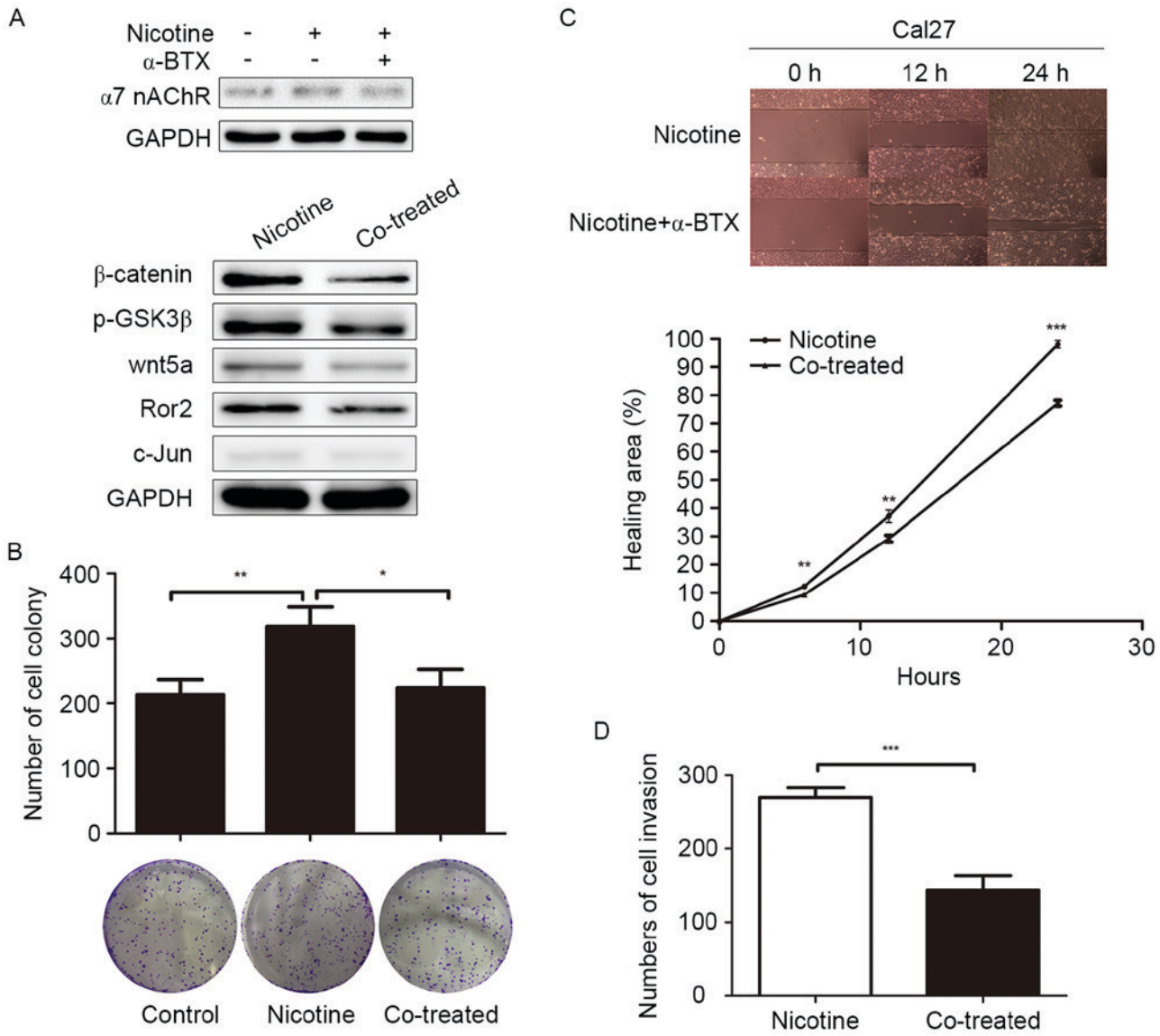

Figure 4. Effect of α-BTX on the nicotine-induced proliferation, migration and invasion of Cal27 cells. (A) α-BTX (0.1 μM) reversed the nicotine-induced upregulation of β-catenin, p-GSK3β, Wnt5a, Ror2 and c-jun in western blot assays. (B) Representative images of a colony-forming assay of Cal27 cells, which were cultured in the presence of nicotine at the indicated concentrations with or without α-BTX (0.1 μM) or in DMSO as a control. (C) Representative images of wound healing of Cal27 in 10 μM nicotine with or without α-BTX (0.1 μM) at indicated time-point. Student's *t*-test was used to compare the speed of wound healing between the nicotine stimulation group and the control group (original magnification, x100). (D) Images of the Transwell invasion of Cal27 after different treatments were analyzed using Image-Pro Plus 6.0 software. The cells were allowed to invade through a layer of Matrigel for 24 h. The number of cells invaded was counted and expressed as the mean ± SD (nicotine group vs. co-treated group: 269.33±14.05 vs. 143.36±20.21, P=0.001) (original magnification, x100) (*P<0.05, **P<0.01, ***P<0.001).

It is well known that metastasis of TSCC is closely related to EMT (28). EMT facilitates tumor cell migration, invasion and metastasis, and may also be a major mechanism of tumor progression (29). Previous findings have demonstrated that a decrease in E-cadherin expression is one of the hallmarks of EMT, which is closely associated with recurrence and survival in TSCC patients (30,31). In addition, previous studies have proved that Wnt/β-catenin pathway is directly connected to EMT in TSCC cells (32,33). In our study, we have found that nicotine can reduce the expression level of E-cadherin and activate Wnt/β-catenin pathway *in vitro*. Combination treatment with nicotine and α-BTX abrogated the increased effects of nicotine-induced migration and invasion in Cal27. Thus EMT involving activation of Wnt/β-catenin pathway may be the molecular mechanisms of nicotine-induced metastasis in Cal27 cells and may be ubiquitous in smoking-related cancers.

The Wnt/β-catenin pathway has been also found to be connected to promote proliferation in non-small cell lung cancer after nicotine stimulation (6,9,10,13,34,35). We first found that nicotine can also activate the Wnt/β-catenin pathway in the TSCC cell line Cal27 *in vitro*. It suggests that nicotine-induce activation of Wnt/β-catenin pathway may be a general molecular signaling effect in different cancer cells. We investigated expression levels of β-catenin in samples of TSCC patients with or without a smoking history. We found that aberrant expression of β-catenin is more common in patients with a smoking history. Based on these findings, nicotine-induce aberrant activation of β-catenin may play a significant role in smoking-related TSCC progression.

Overexpression of Wnt5a has recently been linked to melanoma, osteosarcoma, renal cell carcinoma, prostate cancer, breast cancer and TSCC. Wnt5a is reported to promote invasion of certain types of cancer cells, including HeLa cervical cancer cells, A549 lung cancer cells, and KKLS gastric cancer cells (36). In addition, Ror2 is closely associated with the degree of malignancy in oral epithelial tissue (37). In our study, we found that the expression levels of Wnt5a and Ror2 were increased by nicotine stimulation in Cal27 cells *in vitro*, as well as cell migration and invasion. In addition our results indicate that aberrant expressions of Ror2 and Wnt5a are more common in patients with a smoking history, which are consistent with previous reports (24). According to the results above, we presume that the increased migration and invasion of Cal27 cells after nicotine stimulation may be relevant for the upregulation of Wnt5a and Ror2 which needs further research.

In conclusion, we have demonstrated the effects of nicotine stimulation on Cal27 cells, promoting their proliferation and invasion *in vitro*. Therefore, nicotine may be a possible substance in cigarette smoke that aggressively promotes TSCC and leads to a poor prognosis. We also observed activation of Wnt/β-catenin and upregulation of Ror2 and Wnt5a. These molecular changes may be a common mechanism that induces poor prognosis in other smoking-related cancers. It may be a potential molecular target to slow the development of some smoking-related cancers and improve their prognosis.

Acknowledgements

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