

# Effects of lncRNA ANRIL on proliferation and apoptosis of oral squamous cell carcinoma cells by regulating TGF- $\beta$ /Smad pathway

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**Abstract. – OBJECTIVE:** To investigate the role of the long non-coding ribonucleic acid (lncRNA) antisense non-coding RNA in the INK4 locus (ANRIL) in the proliferation and apoptosis of the oral squamous cell carcinoma (OSCC) cells by regulating the transforming growth factor-beta (TGF- $\beta$ )/Smad pathway.

**PATIENTS AND METHODS:** Human OSCC cells were cultured, and then transfected with small interfering (si)-ANRIL to inhibit the lncRNA ANRIL and ANRIL-OE to overexpress the lncRNA ANRIL. Next, the flow cytometry was carried out to detect the apoptosis rate, the proliferation was determined via methyl thiazolyl tetrazolium (MTT) assay, and the changes in the protein level were detected through Western blotting (WB).

**RESULTS:** The lncRNA ANRIL was highly expressed in the tissues and serum of patients. The proliferation ability of the cells transfected with si-ANRIL was significantly reduced, while that of the cells transfected with ANRIL-OE was overtly increased. The apoptosis rate was (9.21 $\pm$ 5.22)%, (22.3 $\pm$ 1.34)%, and (13.21 $\pm$ 6.22)% in lncRNA ANRIL-OE group, si-ANRIL group and control group, respectively. The protein expression level of the apoptotic protein active caspase-3 was lowered after the treatment with ANRIL-OE, and the key molecules of the TGF- $\beta$ /Smad pathway were notably down-regulated after inhibiting ANRIL with si-ANRIL.

**CONCLUSIONS:** The lncRNA ANRIL regulates the TGF- $\beta$ /Smad signaling pathway to promote the proliferation and suppress the apoptosis of OSCC cells.

*Key Words:*

lncRNA ANRIL, TGF- $\beta$ /Smad pathway, Proliferation, Apoptosis.

## Introduction

Oral cancer, one of the most common head and neck cancer over the world, originates in the oral epithelial cells. Tobacco, alcohol, and betel quid are common carcinogens for laryngeal carcinoma, pharyngeal cancer, and tongue cancer. Pathologically, more than 90% of oral cancer patients have well- or moderately differentiated oral squamous cell carcinoma (OSCC). Some advances have been made in the treatment of OSCC in recent years, but the 5-year survival rate is still less than 50% due to late diagnosis, frequent local recurrence of the primary site and cervical lymph node metastasis after treatment<sup>1,2</sup>. Therefore, the illumination of the molecular pathogenesis of OSCC is conducive to improve the therapeutic effects and design more effective therapeutic strategies. Besides, epidemiological studies have manifested that betel quid is a leading pathogenic factor for OSCC in Southeast Asia and Taiwan<sup>3</sup>. However, the molecular pathogenesis of betel quid chewing-related oral cancer remains unclear.

Long non-coding ribonucleic acids (lncRNAs) play key roles in tumorigenesis. However, their roles and mechanisms in OSCC are largely unknown, especially the antisense non-coding RNA in the INK4 locus (ANRIL). Research has revealed that ANRIL facilitates the proliferation, migration, and invasion of cervical cancer through the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway<sup>4</sup>. Besides, it is further found in a study that ANRIL knockdown inhibits the proliferation and invasion of miR-186, promoting

the apoptosis of cervical cancer cells<sup>5</sup>. In addition, ANRIL is overexpressed in pancreatic cancer, which facilitates epithelial-mesenchymal transition (EMT) by activating the ATM-E2F1 signaling pathway through *in vitro* and *in vivo* studies. Moreover, ANRIL is up-regulated in osteosarcoma, and hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) binds directly to putative hypoxia response elements in the upstream region of ANRIL, further promoting the expression of ANRIL<sup>6</sup>. In prostate cancer, the rs4977574, rs1333048, and rs10757278 polymorphisms of the lncRNA ANRIL are related to benign prostatic hyperplasia and prostate cancer risk<sup>7</sup>. However, the role of ANRIL in the development of OSCC is still unknown.

In this study, it was discovered that ANRIL was overexpressed in OSCC, and the roles of the lncRNA ANRIL in the development and progression of OSCC were analyzed. Based on the data, the lncRNA ANRIL promotes OSCC growth by regulating the transforming growth factor beta (TGF- $\beta$ )/Smad pathway.

## Patients and Methods

### Clinical Specimens

The tissue and serum samples were collected from 56 OSCC patients hospitalized in our hospital from January 2018 to December 2018. All patients were informed of this study that was approved by the Ethics Committee of our hospital.

### Cell Culture and Transfection

OSCC SCC15 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)-F12 (Gibco, Rockville, MD, USA). TW2.6 cells were grown in DMEM mixed with Ham's F12 medium (3:1). The cell lines were purchased from the Cell Bank of the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences (Shanghai, China). The medium was replaced every day. The cells were sub-cultured when the cell confluence reached 80-90%.

The cells were randomly divided into small interfering (si)-ANRIL group (transfected with si-ANRIL), si-TGF- $\beta$ 1 group (transfected with si-TGF- $\beta$ 1), si-ANRIL + si-TGF- $\beta$ 1 group (transfected with si-ANRIL + si-TGF- $\beta$ 1), negative control group (NC group, transfected with NC sequence) and blank group (transfected with no sequence). Before transfection, TPC-1 and SW579 cells were seeded into 6-well culture plates for 1 d ( $5 \times 10^5$  cells/well) to ensure that the confluence reached 80-90% within 24 h. Next, the plasmids and Lip2000 were separately added to 250  $\mu$ L Opti-MEM in accordance with the Lipofectamine<sup>TM</sup> 2000 (Invitrogen, Carlsbad, CA, USA) instructions, followed by still standing at room temperature for 5 min. Then, the plasmids and Lip2000 were uniformly mixed and let stand for 20 min. Thereafter, 500  $\mu$ L plasmid/Lip2000 mixture was dropped into the 6-well culture plate and incubated for 48-72 h for subsequent experiments.

### Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from the cell mixture to be tested according to the specifications of a kit (Promega, Madison, WI, USA). Next, 5  $\mu$ L RNA samples were diluted ( $\times 20$ ) with RNase-free ultrapure water, and an ultraviolet spectrophotometer was used to read the optical density (OD) at 260 and 280 nm to determine the concentration and purity.  $OD_{260}/OD_{280}$  ratio=1.7-2.1 indicated high purity. After that, RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs). An ABI 7500 quantitative PCR instrument (Applied Biosystems, Foster City, CA, USA) was applied in qRT-PCR that was performed under the following conditions: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 34 s, for 40 cycles. The primer sequences synthesized by Shanghai Sangon Biotech (Shanghai, China) are summarized in Table I.

Table I. Primer sequences.

Gene	Forward primer sequence	Reverse primer sequence
LncRNA ANRIL	5'-CCACTCCGCTGTAGTCGTTGTGC-3'	5'-GGTCCCTCATTCCTATCCC-3'
TGF- $\beta$	5'-CTGGAGACTGTAGAGGGATGC-3'	5'-CCTGGCTCCTCACTTGGC-3'
$\beta$ -actin	5'-AAGTACTCCGTGTGGATCGG-3'	5'-ATGCTATCACCTCCCCTGTG-3'

### **Cell Proliferation Assay**

When the confluence reached 80%, the cells were washed twice with phosphate-buffered saline (PBS) following the instructions provided by manufacturers, seeded in a 96-well plate at a density of  $3 \times 10^3$  to  $6 \times 10^3$  (200  $\mu$ L per well, 6 replicate wells), incubated at 37°C and 5% CO<sub>2</sub> for 24-72 h, and added with 20  $\mu$ L methyl thiazolyl tetrazolium (MTT) solution (5 mg/mL, Sigma, St. Louis, MO, USA) to each well. Thereafter, the cells were incubated at 37°C and 5% CO<sub>2</sub> for 4 h, the incubation was terminated, and the medium was discarded. Dimethyl sulfoxide (DMSO; 150  $\mu$ L, Sigma, St. Louis, MO, USA) was added to each well and gently shaken for 10 min to promote the dissolution of crystals. After that, OD value was determined using an enzyme-linked immunosorbent detector at 12 h, 24 h, and 48 h, respectively. MTT curves were plotted with the interval as the X-axis and the OD value as the Y-axis. The assay was repeated three times.

### **Detection of Apoptosis Via Flow Cytometry**

The cells were differently treated and then collected separately. The relative number of Annexin V-positive and/or PI-positive cells was determined through flow cytometry. A flow cytometer (FACScan, BD Biosciences, San Diego, CA, USA) equipped with CellQuest software (BD Biosciences, San Diego, CA, USA) was used to analyze the cells: living cells, dead cells, early apoptotic cells, and late apoptotic cells were distinguished. The percentages of early and late apoptotic cells were compared to those in the control group in each experiment.

### **Western Blotting (WB)**

The cells from each group were separated with trypsin, centrifuged, washed with pre-cooled PBS twice, added with cell lysis buffer, and incubated on ice for protein extraction. A bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China) was employed to determine the concentration of proteins. The equal amounts of proteins were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Billerica, MA, USA). Next, the membrane was immersed in 10% skim milk (in PBS, pH 7.2, containing 0.1% Tween-20) for 2 h and incubated with an appropriate amount

of primary antibody at 4°C overnight, followed by culture with peroxidase-conjugated secondary antibody (KPL, Gaithersburg, MD, USA). Lastly, the detection was conducted via chemiluminescence (Millipore Corporation, Billerica, MA, USA).

### **Statistical Analysis**

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The data were expressed as mean  $\pm$  standard deviation (SD). The homogeneity test of variance was performed, and then, the One-way analysis of variance (ANOVA) was employed for the comparisons among multiple groups, and the Least Significant Difference (LSD) *t*-test was utilized for pairwise comparisons.  $p < 0.05$  indicated that the difference was of statistical significance.

## **Results**

### **Chip Screening Results of Differentially Expressed lncRNAs**

The RNA-seq chip screening technique was applied to analyze 3 cases of OSCC tissues and 3 cases of proliferative oral mucosa tissues, and 978 differentially expressed lncRNAs ( $|\text{fold changes}| \geq 2$ ) were detected in total, of which the lncRNA ANRIL (fold change=6.23) was evidently up-regulated. RNA-Seq data revealed that the lncRNA ANRIL was clearly raised and that the lncRNA ANRIL target genes predicted were significantly enriched in the TGF- $\beta$ /Smad signaling pathway (Figure 1).

### **lncRNA ANRIL Was Highly Expressed in the Tissues and Serum of OSCC Patients**

The qRT-PCR assay was carried out to measure the expression of lncRNA ANRIL in cancer tissues and para-carcinoma tissues of 56 OSCC patients, and it was found that the expression of lncRNA ANRIL in cancer tissues was distinctly higher than that in para-carcinoma tissues ( $p < 0.001$ ) (Figure 2A). Additionally, the expression of lncRNA ANRIL in the serum of these patients was examined via the same assay, and the results showed that the expression of lncRNA ANRIL in OSCC patients was higher than that in healthy controls ( $p < 0.001$ ) (Figure 2B). These results suggest that lncRNA ANRIL may be a potential risk factor for OSCC.

### **LncRNA ANRIL Promoted the Proliferation of OSCC Proliferative Cells**

The OSCC cells were separately transfected with si-ANRIL and ANRIL-OE, and then, the cell proliferation ability was detected. The results (Figure 3) revealed that the proliferation ability of the cells transfected with si-ANRIL was significantly weakened, while that of the cells transfected with ANRIL-OE was overtly strengthened ( $p < 0.05$ ), implying that the lncRNA ANRIL facilitates the proliferation of cells.

### **LncRNA ANRIL Repressed Apoptosis**

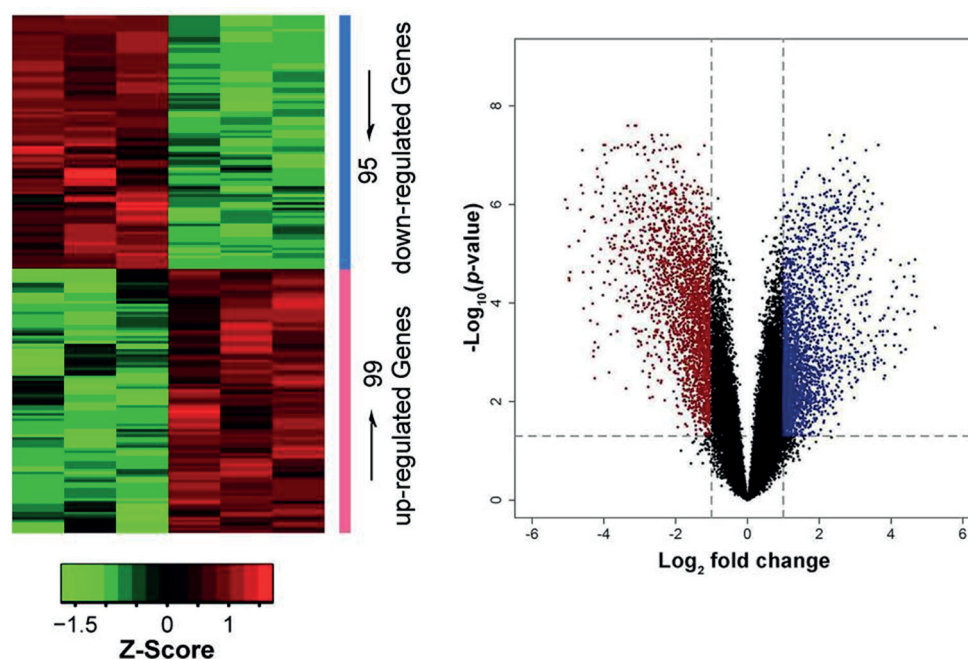
After transfecting cells with si-ANRIL, the apoptosis rate of cells was detected via flow cytometry. The results showed that the apoptosis rate was  $(9.21 \pm 5.22)\%$  in the lncRNA ANRIL-OE group,  $(22.3 \pm 1.34)\%$  in the si-ANRIL group, and  $(13.21 \pm 6.22)\%$  in the control group, showing statistically significant differences ( $p < 0.05$ ) (Figure 4A).

The expression of active caspase-3 in each group of cells was detected after transfection with ANRIL-OE, and it was found that the treatment with ANRIL-OE down-regulated the protein expression level of active caspase-3, an apoptotic protein ( $p < 0.05$ ) (Figure 4B).

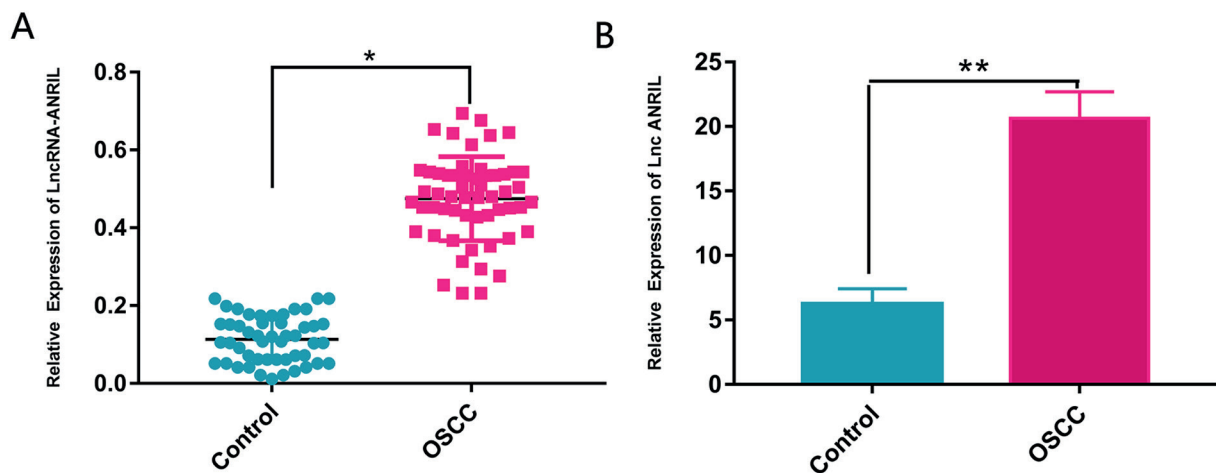
### **LncRNA ANRIL Targeted the TGF- $\beta$ /Smad Pathway to Regulate the Proliferation and Apoptosis of OSCC**

To further clarify the mechanism of lncRNA ANRIL in regulating the proliferation and apoptosis of OSCC by targeting TGF- $\beta$ /Smad, the lncRNA ANRIL was overexpressed, and the pathway screening and the bioinformatics analysis were carried out. The results revealed that the TGF- $\beta$ /Smad pathway was activated after the overexpression of the lncRNA ANRIL (Figure 5).

To further verify that lncRNA ANRIL targeted the TGF- $\beta$ /Smad pathway, the WB was conducted and it was discovered that, after 24 h of transfection with ANRIL siRNA, the expressions of TGF- $\beta$ 1 and phosphorylated (p)-Smad2/3 in the cells were elevated compared with those in the group blank and NC group ( $p < 0.05$ ), while they were clearly lower in the si-TGF- $\beta$ 1 group and si-ANRIL + si-TGF- $\beta$ 1 group than those in the blank group and NC group ( $p < 0.05$ ), and there were no differences in the expressions of TGF- $\beta$ 1 and p-Smad2/3 between the blank group and NC group ( $p > 0.05$ ).



**Figure 1.** Differentially expressed lncRNAs in 3 cases of OSCC tissues and 3 cases of proliferative oral mucosa tissues analyzed via RNA-seq chip screening technology.

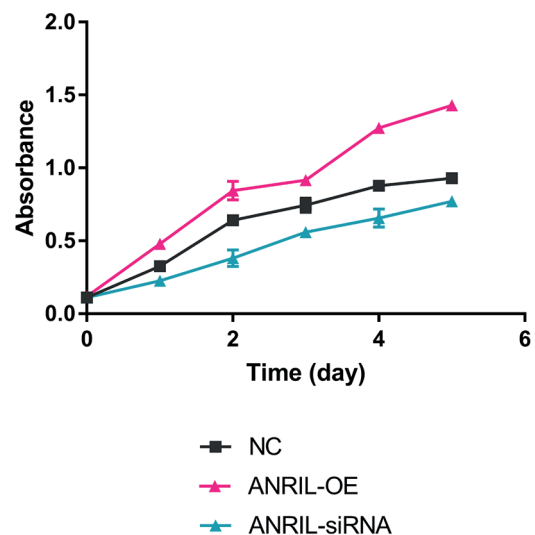


**Figure 2.** *A*, The expression of the lncRNA ANRIL in cancer tissues and para-carcinoma tissues of OSCC patients detected through qRT-PCR. The expression of the lncRNA ANRIL is remarkably higher in patients' cancer tissues than that in para-carcinoma tissues. *B*, The expression of the lncRNA ANRIL in the serum of OSCC patients detected by qRT-PCR. OSCC patients have markedly increased expression of the lncRNA ANRIL in comparison with healthy controls.

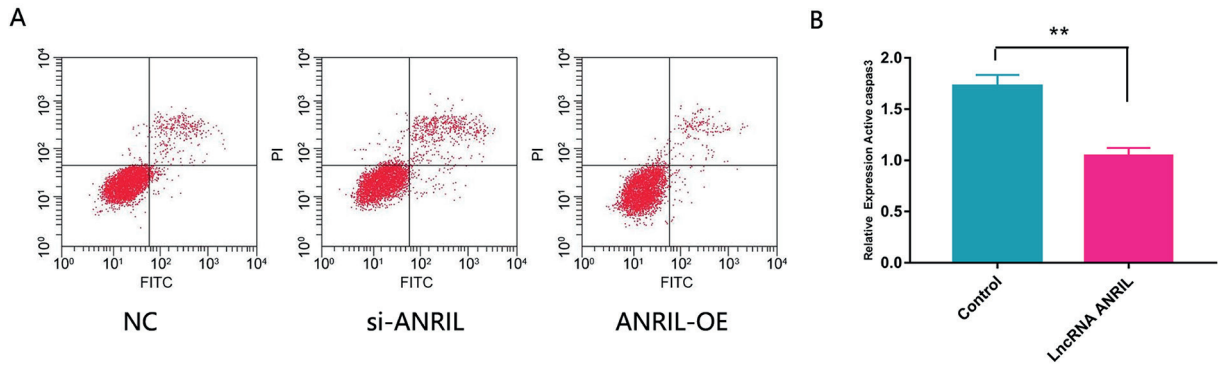
## Discussion

lncRNAs, important non-coding RNAs, participate in the regulation of some cell functions, including apoptosis and invasion, and maintaining induced pluripotent stem cells<sup>8,9</sup>. Besides, they play crucial roles in the epigenetic regulation, transcriptional control, and post-transcriptional regulation, and the RNA-based epigenetic regulatory networks are of great importance in cell and pharmacological evolution<sup>10,11</sup>. ANRIL, also known as CDKN2B-AS, is a lncRNA composed of 19 exons, with a length of 126.3 kb in the genome. It is located in the p15/CDKN2B-p16/CDKN2A-p14/ARF gene cluster in the antisense orientation of the chromosome 9p21<sup>12</sup>. It has been previously proven that ANRIL binds to chromosome 7 (CBX7) and SUZ12, through which it is involved in the repression of the transcription. Moreover, it is reported that the changes in the expression of ANRIL are associated with the development and progression of many tumors<sup>13</sup>. For instance, ANRIL is considered to be a possible oncogene in bladder cancer and positively regulates the proliferation and apoptosis of bladder cancer cells through intrinsic apoptotic pathways. Furthermore, ANRIL is found to be an important player in the progression of esophageal squamous cell carcinoma (ESCC) by suppressing p15 (INK4b) by the TGF- $\beta$ 1 signaling pathway<sup>14</sup>. As for the molecular pathogenesis in thyroid cancer, much progress has been made, and the best proof is that the basic effects of several major signaling

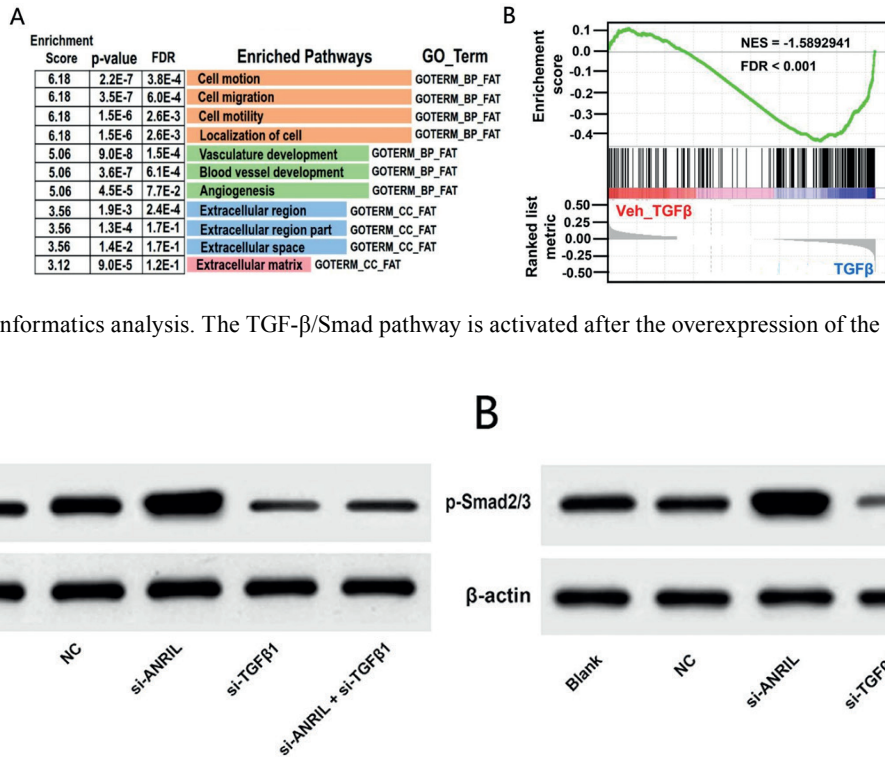
pathways and related molecular disorders have been clarified. TGF- $\beta$  inhibits the proliferation of many human cell lines and tissues (including the thyroid gland), and it has been proposed that the TGF- $\beta$ /Smad signal transduction plays a leading role in inducing EMT in OSCC<sup>15</sup>. Therefore, it was supposed in this study that ANRIL could also exert the functions in the progression of OSCC through the TGF- $\beta$ /Smad signaling pathway.



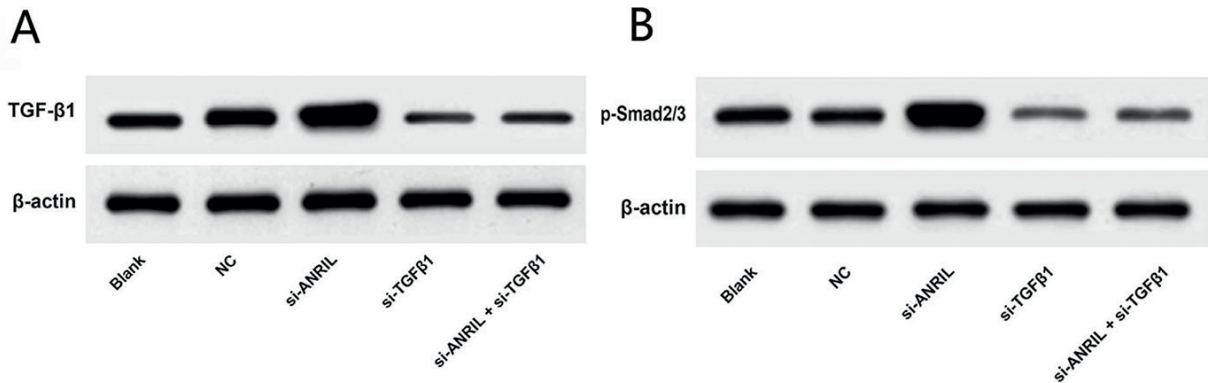
**Figure 3.** Cell proliferation ability detected. The cells transfected with si-ANRIL have significantly weakened proliferation ability, while those transfected with ANRIL-OE exhibit notably strengthened proliferation ability ( $p < 0.05$ ).



**Figure 4.** *A*, Apoptosis rate detected via flow cytometry. The apoptosis rate is (9.21±5.22)%, (22.3±1.34)% and (13.21±6.22)% in lncRNA ANRIL-OE group, si-ANRIL group, and control group, respectively. *B*, The expression level of active caspase-3 in cells in each group determined through Western blotting. The treatment with ANRIL-OE lowers the protein expression level of active caspase-3, an apoptotic protein ( $p < 0.05$ ).



**Figure 5.** Bioinformatics analysis. The TGF-β/Smad pathway is activated after the overexpression of the lncRNA ANRIL.



**Figure 6.** ANRIL exerts functions through the TGF-β/Smad signaling pathway. *A*, The effect of transfection with ANRIL siRNA on the TGF-β1 protein level detected by Western blotting. *B*, The protein expression of p-Smad2/3 in each group of cells detected by WB ( $p < 0.05$ ).

In this study, it was discovered that the lncRNA ANRIL was overexpressed in OSCC, and the knockdown of ANRIL inhibited the proliferation and promoted the apoptosis of OSCC cells. Besides, it was further found that the knockdown

of the lncRNA ANRIL reduced the expression of TGF-β1, repressed the phosphorylation of Smad2, and facilitated the phosphorylation of Smad7, thus inactivating the TGF-β1/Smad signaling pathway. Currently, the correlation between the

lncRNA ANRIL and the TGF- $\beta$  signal transduction pathway is rarely studied. Zhao et al<sup>16</sup> found that the silence of the lncRNA ANRIL increases the expressions of TGF- $\beta$ 1 and p-Smad2/3. ANRIL exerts an inhibitory effect on the TGF- $\beta$  signal transduction pathway, while the overexpression of ANRIL has a different effect, i.e., it activates the TGF- $\beta$  signal transduction pathway in OSCC. Whatcott et al<sup>17</sup> has manifested that the expression of TGF- $\beta$ 1 in OSCC tissues is markedly higher than that in normal tissues and that the up-regulated PlncRNA-1 promotes EMT of OSCC cells by inducing TGF- $\beta$ 1 expression. In oral cancer, TGF- $\beta$  promotes PI3K/AKT signal transduction through p85 $\alpha$  ubiquitination mediated by TRAF6. TMPRSS2/ERG gene fusion activates the TGF- $\beta$  signal transduction by lowering VIM, MMP1, CDH2, and SNAI2 and triggers the EMT of human OSCC cells<sup>18</sup>. Smad7 is normally regulated by the degradation mediated by the ubiquitination of Smurf2 and ITCH, however, the regulation on its phosphorylation remains to be explored. Zhang et al<sup>19</sup> reported that the silence of the ANRIL expression represses the proliferation and invasion of different cancer cells and induces apoptosis. ANRIL is consumed by transfection with ANRIL siRNA, thereby resulting in arrest in cell cycle G2/M phase, which led to the inhibition of cancer cell proliferation by modulating p15 and other G2/M phase control-related genes. ANRIL is transcribed in the opposite direction of the INK4b-ARF-INK4a gene cluster encoding three tumor suppressors: p15 (INK4b), p14 (ARF), and p16 (INK4a). Moreover, it is suggested that ANRIL inhibits the expression of p15 (INK4b), p14 (ARF), and p16 (INK4a)<sup>20,21</sup>. It was reported in this study for the first time that the lncRNA ANRIL positively regulated the TGF- $\beta$ 1/Smad signaling pathway in OSCC. The specific mechanism needs to be further investigated.

## Conclusions

We proved in this study that the silence of ANRIL, a key lncRNA, represses the invasion and metastasis of OSCC cells. In addition, ANRIL can inhibit the expression of TGF- $\beta$ /Smad signaling pathway and reduce the expression of p15 (INK4b), thus promoting the invasion and metastasis of OSCC cells, which provides new insight into the role of the lncRNA ANRIL in tumorigenesis.

## Conflict of Interests

The Authors declare that they have no conflict of interests.

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