Knowdown of lncRNA HOXA-AS2 inhibits proliferation, invasion and migration of oral squamous cell carcinoma

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Research

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Abstract

Background

Oral squamous cell carcinoma (OSCC) is one of the most common cancers in the world. The aim to the study was to evaluated the biological function and partly underlying regulatory mechanism of lncRNA homeobox A cluster antisense RNA2 (HOXA-AS2) on oral squamous cell carcinoma.

Methods

The expression of HOXA-AS2 in OSCC cells was detected by quantitative real time polymerase chain reaction (qRT-PCR). HOXA-AS2 expression was modified by transfection with HOXA-AS2 knockdown into TCA-8113 cells. The biological activity of TCA-8113 cells were detected by Cell Counting Kit-8 (CCK-8), EdU staining, Tunel staining, flow cytometry, wound healing, transwell assay and western blot. The relationship between HOXA-AS2 and EZH2 was analyzed by RNA immunoprecipitation (RIP).

Results

At first, in this study, HOXA-AS2 expression in TCA-8113 cell line was increased compared with normal oral cells. Furthermore, HOXA-AS2 knockdown could inhibit cell viability, migration and invasion. Besides, EZH2 is the target of HOXA-AS2 in TCA-8113 cells. EZH2 expression was reduced by the HOXA-AS2 knockdown and the expression of P21 was negatively correlated to the expression of HOXA-AS2 in TCA-8113 cells.

Conclusion

In this study, silencing HOXA-AS2 reduced cell viability, invasion and migration capacity and EZH2, as an oncogene, could be downregulated by HOXA-AS2 knockdown in OSCC cells.

Background

Oral squamous cell carcinoma (OSCC) is a common malignant tumor of the head and neck, accounting for about 5% of all malignant tumors, which is prone to cell invasion and migration (1). Related Studies showed that the prevalence rate has increased year by year in recent years and its 5-year survival rate is only 41%-79.5% (2). Because the occurrence and development of OSCC involves a wide variety of biological processes, exploring the mechanism is of great significance for OSCC targeted therapy. At present, although most OSCC are treated by surgical resection, radiotherapy and chemotherapy, the prognosis is poor and serious side effects are easy to occur. Therefore, a more urgent task is to explore the molecular mechanism of oral cancer in order to find new therapeutic targets and new diagnostic markers.

Long non-coding RNA (lncRNA) is a type of non-protein coding transcript with a length of more than 200 bp. Accumulating evidences have shown that the biological function of lncRNA is complex, which is closely related to the proliferation, invasion, metastasis and recurrence of tumor cells (3, 4). For example, Gao *et al*/ demonstrated that the expression level of lncRNA was related to TNM stage and lymph node metastasis in patients with tongue squamous cell carcinoma, which confirmed that the abnormal regulation of lncRNA was related to the biological activity of tongue squamous cell carcinoma (5). Meanwhile, Fang *et al*/ proved that lncRNA UCA1 could promote the proliferation and cisplatin resistance of OSSC by down-regulating miR-184 expression, suggesting that targeting UCA1 may be a potential therapeutic strategy for patients with OSSC (6). In addition, recent study has shown that lncRNA PLAC2 can be transcribed and activated by H3K27ac modification in the promoter region of oral squamous cell carcinoma, and promote cell growth and migration by activating Wnt/ β -catenin signaling pathway (7). Previous studies have indicated that lncRNA HOXA-AS2 could show different expressions in various malignant tumors, and its carcinogenicity mainly suppresses or promotes the expression of related genes directly or indirectly (8–11). However, there are few studies on the relationship between HOXA-AS2 and OSSC. Therefore, the purpose of this study is to detect the expression of lncRNA HOXA-AS2 in oral cancer cells and analyze the the biological functions.

EZH2 is a new human gene discovered in 1996. It was initially found that the gene is located on the human chromosome 21q22.2 and related to the Down syndrome. Some studies have proved that lncRNA-HOXA-AS2/EZH2/LSD1 oncogene complex could promote the proliferation in pancreatic cancer cells (12). However, the expression mechanism of EZH2 in OSSC is not clear.

The aim of the study is to evaluate the relationship between HOXA-AS2 and the proliferation, migration and invasion on OSSC. In the study, we found that the expression of HOXA-AS2 was significant higher in OSSC. Moreover, silencing HOXA-AS2 reduced the activity of oral cancer cells and affect the expression of EZH2. Our results provide a new evidence for revealing that HOXA-AS2 may be a new clinical strategy for the treatment of oral squamous cell carcinoma.

Materials And Methods

Cell culture

HIOEC cells were purchased from BeNa Culture Collection (BNCC, Beijing, China). CAL-27, TCA-8113, SCC-4, SCC-9 and SCC-15 cells were all obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HIOEC cells were cultured in Dulbecco's modified Eagle's medium: nutrient Mixture F-12 (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Other cells were cultured in DMEM medium containing 10% FBS, 100U/mL penicillin and 100U/mL streptomycin. All the cell lines were incubated in a fully humidified atmosphere with 5% carbon dioxide at 37°C. Change the medium regularly and observe the growth of cells on time. Cell passage are carried out when the cell confluence reach to 90%. Logarithmic phase cells were taken for follow-up experiment.

Cell transfection

Short-hairpin RNA (shRNA) against HOXA-AS2 (shRNA HOXA-AS2-1, shRNA HOXA-AS2-2) and corresponding empty vector (shRNA NC) were purchased from GenePharma (Shanghai, China). Liposome 3000 transfection reagent (Thermo Fisher Scientific) were used to transfect into TCA-8113 cells following the product instruction. After 48 hours, qRT-PCR was used to detect.

qRT-PCR analysis

Total RNA was extracted by Trizol. The optical density (OD) at 260 nm and 280 nm was measured by ultraviolet spectrophotometer respectively. Meanwhile, the concentration and purity of RNA were calculated. According to the All-in-One™ qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA) instruction, RNA was reverse transcribed into cDNA and examined by the qRT-PCR. GAPDH was taken as the internal reference, the relative expression of target gene was calculated by $2^{-\Delta\Delta C_t}$ method.

CCK-8 assay

Cells in the logarithmic growth period were seeded into 96-well plate at a density of 2×10^3 /well for routine culture. After 24 h, 48 h, 72 h and 96 h, 10 μ L CCK-8 solution (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) was added to each well and incubated continued for 2 h. The absorbance of each well was measured by a microplate reader (Bio-Rad, Hercules, CA, USA) at 450 nm wavelength.

EdU staining assay

Cells concentration were adjusted to 1×10^5 cells/ml, seeded into 96-well plates and cultured continued overnight at 37°C in 5% carbon dioxide. According to the EdU immunofluorescence staining kit (Ribobio) instructions, the concentration of EdU solution was adjusted to 10 μ M and 100 μ L diluted solution was added to each well. Culture medium was discarded after incubation for 4 hours and cleaned with PBS twice. Cells were fixed with paraformaldehyde and stained with Apollo. After staining was completed, observe and take pictures under a fluorescence microscope (Olympus BX 60 fluorescence microscope, Japan).

Tunel staining assay

The apoptotic cells were stained according to the operation of the TUNEL staining kit (Nanjing KeyGen Biotech, Co., Ltd., Nanjing, Jiangsu, China) and were observed by phase-contrast microscopy. Apoptotic nuclei were stained brownish yellow.

Flow cytometry analysis

Cell-cycle distribution was detected by flow cytometry (FACScan, Becton 4 Dickinson). Apoptosis detection refers to the instruction manual of the Annexin V-FITC Apoptosis Detection Kit (Sangon, China). Treated cells were diluted into 1×10^6 cells/ml suspension and resuspended in 500 μ L buffer solution. 5 μ L PI and 5 μ L Annexin V-FITC were added to incubate 30 min in the dark at room temperature. Analysis of apoptotic cells was performed by flow cytometry.

Cell migration and invasion assay

The ability of cell migration was analyzed by a wound scratch test. According to the experimental requirements, cells were seeded into 6-well plates, after the cells grew to complete fusion, the layer of cells was scratched to form wounds using a sterile 20 μ l pipette tip and cultured for 48 hours. The image of the scratched area was captured under the phase contrast microscope and the cell healing area was analyzed by ImageJ software (NIH, USA). Transwell invasion assay was used to evaluate cell invasion. 8 μ m transwell chamber (Corning, cat:354578) was applied to this experiment. Apply 5 μ l Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA) to the chamber and adjust the cell concentration, then continuing to culture normally for 4 hours, the excess cells in the inner side of the chamber were washed with PBS twice, fixed with polyformaldehyde. Finally, the images were observed by the inverted microscope (Leica).

RNA immunoprecipitation (RIP) assay

RNA immunoprecipitation assay was conducted using the Magna RIP RNA-Binding Protein Immunoprecipitation kit (EMD Millipore) following the manufacturer's protocols. RIP was treated with anti-EZH2 antibody (Abcam, Cambridge, MA, USA) and IgG was used as control. Coprecipitated RNAs were detected by qRT-PCR.

Western blot analysis

Total protein was extracted with RIPA lysis and protein concentrations were determined with a Pierce® BCA Protein Assay Kit (Thermo). Proteins on the SDS-PAGE gel were transferred to the PVDF membrane (Millipore, USA) and 5% skimmed milk powder was sealed for 1 h at 37°C. These membranes were treated with primary antibodies against CDK2 (1:1000; cat. no. #2546; Cell Signaling Technology Inc., USA), cyclinE1 (1:1000; cat. no. #20808; Cell Signaling Technology Inc., USA), P27 (1:1000; cat. no. #3686; Cell Signaling Technology Inc., USA), MMP-2 (1:1000; cat. no. #40994; Cell Signaling Technology Inc., USA), MMP-9 (1:1000; cat. no. #13667; Cell Signaling Technology Inc., USA), Bcl-2 (1:1000; cat. no. #3498; Cell Signaling Technology Inc., USA), Bax (1:1000; cat. no. #5023; Cell Signaling Technology Inc., USA), cleaved caspase-3 (1:1000; cat. no. #9661; Cell Signaling Technology Inc., USA), P21 (1:1000; cat. no. #2947; Cell Signaling Technology Inc., USA) and GAPDH (1:1000; cat. no. #5174; Cell Signaling Technology Inc., USA) at 4 °C overnight, respectively. Blots were then washed 3 $\times$ 10 min with TBST and incubated with secondary antibodies conjugated to horseradish peroxidase (1:500; sc2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature. The immunoreactive bands were detected using the ECL kit (GE Healthcare). GAPDH was taken as an internal reference.

Statistical analysis

All data analyses were performed using GraphPad Prism6.0 software (GraphPad Software Inc., San Diego, CA, USA). The data were expressed as mean $\pm$ standard deviation (SD) and the differences between samples were analyzed by Student's t test or one-way ANOVA. $P < 0.05$ indicated that the difference was statistically significant. All experiments were carried out for three times or more, independently.

Results

HOXA-AS2 was upregulated in OSSC cell lines

Firstly, we detected the expressions of HOXA-AS2 in five OSSC cell lines (CAL-27, TCA-8113, SCC-4, SCC-9 and SCC-15) and one normal oral epithelial cell line (HIOEC). As shown in Fig. 1, the expressions of HOXA-AS2 in OSSC cell lines were significantly increased. Of those, TCA-8113 was the most highly expressed. Therefore, we chose TCA-8113 cells for the subsequent experiments.

Knockdown of HOXA-AS2 decreased cell viability

We constructed two interfering plasmids targeting HOXA-AS2 and detected the levels of interference. As shown in Fig. 2A, HOXA-AS2 protein expression levels were lowest in the cells transduced with shRNA-HOXA-AS-1. Subsequently, we determined the effects of shRNA-HOXA-AS2-1 on cellular viability. The results of CCK-8 assay showed that there was no significant difference in cell viability between each group at 24 h. The cell viability in shRNA-HOXA-AS2-1 group was significantly lower than that in control group and empty plasmid group at 48 h and 72 h (Fig. 2B). Edu results showed that there was no significant difference in green fluorescence intensities between the empty plasmid group and the control group, but the fluorescence intensities in the shRNA-HOXA-AS2-1 group was significantly lower than that in the control group (Fig. 2C). Taken together, knockdown of HOXA-AS2 reduced cell viability.

Knockdown of HOXA-AS2 inhibited the proliferation and induces cell cycle arrest of TCA-8113 cells

We then studied whether HOXA-AS2 knockdown could affect the progression of TCA-8113 cell cycle. As shown in Figs. 3A and 3B, compared with the control group, shRNA-HOXA-AS2-1 significantly increased the proportion of cells in G0/G1 phase and decreased the proportion of cells in S phase and G2 phase. This indicated that the knockdown of HOXA-AS2 resulted in cell cycle arrest of TCA-8113 cells at the G1/S transition. The expressions of cell proliferation-related proteins were detected by Western blot. The results showed that HOXA-AS2 knockdown significantly decreased the expression of CDK2 and cyclinE1, but increased the expression of P27 (Fig. 3C and 3D). Therefore, knockdown of HOXA-AS2 inhibited the proliferation and induces cell cycle arrest of TCA-8113 cells.

Knockdown of HOXA-AS2 inhibited cell migration and invasion in TCA-8113 cells

We detected the effects of HOXA-AS2 knockdown on the migration and invasion ability of TCA-8113 cells by wound healing assay and transwell assay. In the migration experiment, as shown in Fig. 4A and 4C, the cell density of HOXA-AS2 knockdown at 24 h was lower than that of the control group, and the difference was statistically significant. In the invasion test, as shown in figure Fig. 4B and 4D, shRNA-HOXA-AS2-1 significantly decreased the average number of cells penetrating the perforated membrane compared with the control group. In addition, we detected the expressions of MMP-2 and MMP-9 by Western blot. As shown in Fig. 4E and 4F, HOXA-AS2 knockdown significantly reduced the expressions of

MMP-2 and MMP-9. Taken together, knockdown of HOXA-AS2 inhibited cell migration and invasion in TCA-8113 cells.

Knockdown of HOXA-AS2 increased apoptosis in TCA-8113 cells

Apoptosis was detected by TUNEL staining. The cells were observed under a fluorescent microcopy. The positive signal of Tunel was displayed as yellow fluorescence and most of them were apoptotic cells in morphology. 48 h after transfection, the number of apoptotic cells in shRNA-HOXA-AS2-1 group was significantly higher than that in the control group (Fig. 5A). In addition, we examined the pro- and anti-apoptotic protein expressions by Western blot. As shown in Fig. 5B and 5C, the results showed that HOXA-AS2 knockdown significantly decreased the expression of Bcl-2 and increased the expressions of Bax and cleaved caspase-3. Taken together, knockdown of HOXA-AS2 increased apoptosis in TCA-8113 cells

HOXA-AS2 knockdown suppressed the expression of EZH2.

A previous study found that EZH2 often functions as an inhibitor in cancer suppressor genes, and EZH2 could combine with lncRNA to regulate the expression of P21 in tumors. Therefore, we detected the relationship between EZH2 and HOXA-AS2 by RIP. The experimental results showed that HOXA-AS2 could target EZH2 (Fig. 6A). In addition, we also determined the correlation between HOXA-AS2 and EZH2 or P21. As shown in Fig. 6B and 6C, HOXA-AS2 knockdown significantly increased the expression of P21 while decreased the expression of EZH2.

Discussion

In this study, we found that the expression of HOXA-AS2 was enhanced in oral cancer cell lines, and its knockdown treatment could effectively inhibit the proliferation, migration and invasion of cancer cells. In addition, we also found that HOXA-AS2 could bind to the oncogene EZH2 and show a positive correlation, which indicated that the tumor suppressor mechanism of HOXA-AS2 knockdown may be mediated by down-regulation of EZH2, but further functional experiments are needed to verify this hypothesis. This study provides a new potential therapeutic target for the treatment of OSSC.

OSSC is a common disease in oral oncology department, which is prone to lymph node metastasis, resulting in poor prognosis and low survival rate. Traditional surgical treatment can not meet the needs of patients. It is reported that the occurrence and development of OSSC is a complex biological process involving multiple genetic and epigenetic changes (13). With the continuous development of bioinformatics and genomics, studies have confirmed that a group of lncRNAs with length more than 200nt and non-coding ability are abnormally expressed in OSSC. Abnormal expression of lncRNAs can act

as different types of functional molecules and play a regulatory role in cell growth, proliferation, migration, invasion and other biological behaviors (14–16).

HOXA-AS2 is the lncRNA of 1048-bp located between HOXA3 and HOXA4 genes in the HOXA cluster, is a promising candidate sequence among all tumor-related lncRNAs. The expression of HOXA-AS2 in gastric cancer tissues was higher than their matched normal tissues and the G1 phase arrest and apoptosis of gastric cancer cells was promoted by silencing HOXA-AS (17). Down-regulation of HOXA-AS2 in colorectal cancer induced G0/G1 cell cycle arrest and reduce the number of cells in S phase (18). Up-regulation of HOXA-AS2 in breast cancer is associated with tumor invasion, lymph node metastasis and postoperative survival (19). In summary, HOXA-AS2 were highly expressed in most tumor cells, but little is known in oral cancer cells. In this study, we selected five kinds of OSSC cells for experiments, the results showed that compared with normal cells, the expressions of HOXA-AS2 were all remarkably up-regulated, of which TCA-8113 cells increased most significantly, which was selected for the next experiments. The results of CCK-8, Edu staining, cell scratch, migration, invasion and apoptosis experiments showed that HOXA-AS2 knockdown could effectively inhibit the proliferation, migration and invasion of cancer cells, promote apoptosis, arrest the cells in transition from G0/G1 phase to S phase. However, its specific mechanism is not clear.

EZH2 is a kind of oncogene, as a core member of the polycomb group (pcG), which can regulate the silencing of downstream genes through the trimethylation of histone 3 lysine 27 (H3K27me3). As an oncogene, it plays an important role in the regulation of cell proliferation, differentiation, migration and tumorigenesis (20–23). We found that HOXA-AS2 could interact with EZH2 through bioinformatics software and RIP experiments verified that HOXA-AS2 could target EZH2. Furthermore, silencing HOXA-AS2 significantly downregulated the expression of EZH2 and upregulated the expression of P21. As an important regulatory factor of cell cycle, P21 protein is closely related to many kinds of cancer occurrence and development (24, 25). We speculate that HOXA-AS2 may affect the tumor growth by regulating the expression of EZH2, but the specific mechanism of HOXA-AS2 in OSSC remains to be studied further.

Conclusion

In conclusion, Our study proved that the expressions of HOXA-AS2 were significantly up-regulated in oral cancer cells and HOXA-AS2 knockdown could effectively inhibit cell proliferation, migration and invasion, which confirmed the therapeutic effect of HOXA-AS2 knockdown on OSSC. In addition, HOXA-AS2 may promote tumor growth by regulating EZH2, but the specific mechanism remains to be further studied.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

Not applicable.

Funding

Not applicable.

Authors' contributions

Zhen Zhao, Yan Xing and Shanghua Jing made substantial contributions to the conception and design of the present study. Zhen Zhao and Yan Xing designed the research; Zhen Zhao, Yan Xing, Fei Yang, Zhijun Zhao, Yupeng Shen and Junjian Song performed the research; Zhen Zhao and Yan Xing analyzed the data and wrote the paper.

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Figures

Figure 1

HOXA-AS2 was upregulated in OSSC cell lines. Data are expressed as the mean $\pm$ standard deviation (n=3). ***p < 0.001 vs. HIOEC.

Figure 2

Knockdown of HOXA-AS2 decreased cell viability. A. Interference efficiency detection by qRT-PCR. *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. -NC. B. Proliferation in each group of cells after transfection was detected using CCK-8 kit. *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. shRNA. C. Cell proliferation was determined using the EdU assay.

Figure 3

Knockdown of HOXA-AS2 inhibited the proliferation and induces cell cycle arrest of TCA-8113 cells. A. Cell cycle was detected by flow cytometry analysis. B. Quantitative analysis of cell cycle distribution. *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. shRNA-NC. C. Protein bands in the image. D. Relative protein levels of CDK2, cyclinE1 and P27 in different groups, GAPDH was used as the control. *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. shRNA.

Figure 4

Knockdown of HOXA-AS2 inhibited cell migration and invasion in TCA-8113 cells. A. Cell migration was determined by a scratch assay. B. Detecting cell invasion by Transwell invasion assay. C. Quantitative analysis of migrated rate. D. Quantitative analysis of invasive rate. E. Protein bands in the image. F. Relative protein levels of MMP-2 and MMP-9 in different groups, GAPDH was used as the control. *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. shRNA.

Figure 5

Knockdown of HOXA-AS2 increased apoptosis in TCA-8113 cells. A. TUNEL detection of apoptosis. B. Protein bands in the image. C. Relative protein levels of Bcl-2, Bax and cleaved caspase-3 in different groups, GAPDH was used as the control. *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. shRNA.

Figure 6

HOXA-AS2 knockdown suppressed the expression of EZH2. A. RNA immunoprecipitation (RIP) demonstrating specific binding of HOXA-AS2 to EZH2 mRNA. B. Protein blot. C. Relative protein levels of P21 in different groups, GAPDH was used as the control. D. Relative protein levels of EZH2 in different groups, GAPDH was used as the control. *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. shRNA.