Original Article

Study on the mechanism of SALL4 down-regulation in promoting the invasion and migration of oral squamous cell carcinoma and influencing the survival and prognosis of patients

Jin Li[1], Bin Zhang[2], Rongchen Xu[1], Bingxin Wang[1], Wenjun Hao[3], Xiaoxiao Zhang[4]

[1]Department of Stomatology, The Third Medical Center of Chinese PLA General Hospital, Beijing, China; [2]Department of Stomatology, The First Medical Center of Chinese PLA General Hospital, Beijing, China; [3]Department of Stomatology, The First Retired Cadre Rest House in Dongcheng, Beijing Garrison of the Chinese People's Liberation Army, Beijing, China; [4]Department of Information Section, The Third Medical Center of Chinese PLA General Hospital, Beijing, China

Contributions: (I) Conception and design: J Li; (II) Administrative support: B Zhang; (III) Provision of study materials or patients: R Xu; (IV) Collection and assembly of data: B Wang; (V) Data analysis and interpretation: W Hao, X Zhang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Bin Zhang. Doctor, Deputy Chief Physician, Department of Stomatology, The First Medical Center of Chinese PLA General Hospital, No. 28 Fuxing Road, Haidian District, Beijing, China. Email: doctorzbin@163.com.

Background: To investigate the identification of spalt-like transcription factor 4 (SALL4) in oral squamous cell carcinoma (OSCC).

Methods: Recombinant cells loaded with miRNA expression cells were used to transform Tca8113 cells. Simple Tca8113 cells were used as the control group. We detected SALL4 messenger RNA (mRNA) before and after transfection by reverse transcription polymerase chain reaction (RT-PCR) and protein immunoblotting (western blot) A and protein expression. A dual luciferase reporter system was used to verify the targeted regulation of SALL4 and identify miRNA-S to test the effect of miRNA related to SALL4 regulation on the invasion and metastatic ability of Tca8113 cells.

Results: The expression of SALL4 mRNA in Tca8113 cells was higher than that in the downregulated and control groups, respectively ($P<0.05$); there was no difference in Tca8113 cells between the upregulated and downregulated groups ($P>0.05$). Dual luciferase reporter system showed that the identified miRNA was miRNA-S; there were no differences in migration and invasion of Tca8113 cells between the up- and down-regulated groups ($P>0.05$).

Conclusions: In human OSCC, SALL4 regulation-related miRNAs are poorly expressed and can inhibit the invasion and metastasis of tumor cells, which is expected to become a new therapeutic target for OSCC.

Keywords: Spalt-like transcription factor 4 (SALL4); invasion and migration; oral squamous cell carcinoma (OSCC); prognosis of survival

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignant tumors worldwide. The number of new OSCC cases in China comprises 50.0% of the cases globally, ranking second among malignant tumors in China (1). At present, OSCC is mainly treated with surgical resection to delay the development of the disease. With the advancement of surgical technology and perioperative treatment level, the success rate of OSCC has been improved (2). However, the 5-year survival rate of patients with OSCC is only 40–50%, which may be related to the recurrence and metastasis of tumors after surgery. Previous clinical studies

(3-5) have shown that epithelial to mesenchymal transition (EMT) plays an important role in initiating epithelial-derived tumors. During EMT, E cadherin, keratin, and other proteins show low expression, while N cadherin and vimentin are highly expressed, along with the disease prognosis. With the continuous development of EMT, cells will deviate from cell proliferation and undergo distant metastasis (6).

Spalt-like transcription factor 4 (*SALL4*) is a homologous gene of *Drosophila Spalt* (7-9). There are four members of this family, namely *SALL1*, *SALL2*, *SALL3*, and *SALL4* (10). In the past few decades, several stem cell-related genes have been used as markers for tumor diagnosis, and the level of gene expression is important for prognosis. The *SALL4* gene forms a regulatory network with other stem cell-related genes (such as *OCT-4*, *NANOG*, *Sox2*, etc.) and plays a crucial role in embryonic development and formation (11). Mutation of the *SALL4* gene can lead to the occurrence of a variety of genetic diseases (12). For example, there are many autosomal dominant genetic diseases: Duane-radial ray syndrome (DRRS), Holt-Oram syndrome (HOS), IVIC syndrome, and thalidomide fetal malformation. Although SALL4 is expressed in human fetal liver, kidney, and intestines, its expression gradually decreases or even silences with maturation. As a proto-oncogene, the high expression of *SALL4* can lead to the development of a variety of malignant tumors (13-15). Currently, it is known that *SALL4* is highly expressed in B lymphoblastic lymphoma, acute and chronic myeloid leukemia (16). Although the SALL4 was discovered some time ago, a study had been conducted on it until its further identification as a possible marker and molecular target for prognosis (17). It was first studied in germinal cell tumors, and the results showed that *SALL4* is a sensitive and effective tumor marker. Subsequently, it was found that high expression of *SALL4* in digestive system tumors is closely associated with poor prognosis. Many studies (18,19) have been carried out on *SALL4* in a range of tumors, but so far, there have been no reports on the expression and role of *SALL4* in OSCC. That may affect the prognosis of patients with tongue squamous carcinoma clinical pathologic factors including age, sex, pathologic stage, lymph node metastasis, tumor size, tumor invasion the midline, clinical staging, surgical treatment of single factor analysis, it is concluded that there are statistically significant clinical pathologic factors are: the middle route of the pathologic stage, lymph node metastasis, tumor invasion, surgery.

In order to further determine the role of SALL4-regulated miRNAs in OSCC, the dual luciferase reporting system was used to further verify the targeted regulation of miRNA on SALL4, and the identified miRNAs were recorded as mirNA-S. There was no significant difference in the number of migration and invasion cells in up-regulation group or down-regulation group ($P>0.05$). The number of migration and invasion cells in both up-regulated and down-regulated groups was lower than that in the control group ($P<0.05$), suggesting that mirNA-S expression is the main expression of SALL4-regulated miRNAs in OSCC, which can inhibit the proliferation and growth of oral squamous cells, thus delaying the development of the disease.

SALL4 is a transcription factor containing zinc finger structure, which is specifically expressed in primordial germ cell tumors and plays an important role in early embryonic development. Previous studies (20,21) have shown that the *SALL4* initiator is a STAT3-binding region, which can activate cell self-renewal and regulate the function of embryo-fetal stem cells. Its conduction pathway is closely related to the expression of tumor cells, but its specific mechanism and role in tumor metastasis have not been clarified. Tongue tissue has a strong muscularization structure and abundant lymphatic network, which leads to tongue tissue is more vulnerable to invasion and metastasis. Tongue squamous cell carcinoma is more prone to lymph node metastasis than any other oral cancer. Lymph node metastasis is an important factor affecting the prognosis of patients with tongue squamous cell carcinoma. Patients with lymph node metastasis have a high local recurrence rate and poor prognosis. Compared with previous studies, this study continued to further explore the mechanism of SALL4 in the occurrence and development of human OSCC, and carried out cell and other related experiments to verify it. Therefore, in this study, the cell control method was adopted to assist the identification of miRNAs regulated by *SALL4* in OSCC and its mechanism of inhibiting tumor metastasis. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2974/rc).

Methods

Cell culture

We purchased OSCC Tca8113 cell lines from the Cell Resource Center, Chinese Academy of Sciences (Shanghai, China). The OSCC Tca8113 cells were seeded in 10% fetal bovine serum (FBS) Roswell Park Memorial Institute

(RMPI) 1640 medium, and cultured in a 37 ℃, 5% CO_2 cell incubator. The cells were sub-cultured after reaching 80% confluence, and generation 2 logarithmically grown cells were set aside for future use.

Main reagents and instruments

Blue streptomycin FBS, Dulbecco's modified Eagle medium (DMEM) and high sugar medium (HyClone Laboratories, Logan, UT, USA) β-actin antibody (Santa Cruz, Santa Cruz, CA, USA), Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), SALL4 RNA Reverse Transcription Kit (Ambion, Waltham, MA, USA), gel imaging analysis system (Baygene Biotech, Beijing, China), Cell culture Plate (Gibco).

Polymerase chain reaction experiment

The expression of miRNA in Tca813 cells was up-regulated and down-regulated by polymerase chain reaction; simple Tca8113 cells were used as the control group. The treated cells were thoroughly mixed with TRIzol 500 μL (Thermo Fisher Scientific, Waltham, MA, USA), and 0.2 mL chloroform was added. The cells were shaken violently for 15 seconds, stood at room temperature for 2–3 minutes, and centrifuged at 3,500 r/min for 15 minutes. The RNA was precipitated, transferred to a new EP tube, 0.5 mL isopropyl alcohol was added, mixed evenly, placed in the refrigerator at –20 ℃, and centrifuged at 1,194 g for 10 minutes. Diethyl pyrocarbonate (DEPC) 250 μL and ethyl alcohol 750 μL were added to the precipitation, centrifuged at 4,500 r/min for 5 minutes, and the precipitation was dried on a workbench for 20 minutes. The concentration of RNA was detected by ultraviolet spectrophotometer and RNA purification was completed (RNase was used as blank control), and the absorbance value was measured under A260. We used DNase to treat RNA and placed it into refrigerator after treatment. Before and after transfection, the mRNA level of *SALL4* reached water level by reverse transcription (RT)-PCR. The PCR reaction bars were set as 30 ℃, 10 minutes, 42 ℃, 30 minutes, 99 ℃, 5 minutes, 5 ℃, 5 minutes for 35 consecutive cycles, and it was prolonged at 72 ℃ for 10 minutes.

Western blot experiment

The OSCC Tca81 13 cells were washed with phosphate-buffered saline (PBS) 3 times, followed by ice lysis for 30 minutes with radioimmunoprecipitation assay (RIPA), centrifuged at 2,500 r/min at 4 ℃ for 30 minutes, and the supernatant was collected. The protein content was determined by immunohistochemical (IHC) method (bicinchoninic acid; BCA). The proteins were isolated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), centrifuged at 1,000 r/min for 20 minutes, and electrically transferred to a polyvinylidene fluoride (PVDF) membrane for 2 hours. Then, SALL4 protein primary antibody was added and incubated at 4 ℃ overnight. The membrane was washed 5 times in tris-buffered saline with Tween 20 (TBST), for 5 minutes/time. The SALL4 protein secondary antibody was added for continuous incubation for 2 hours, overnight. The membrane was then washed 5 times, for 5 minutes/time in TBST, a little enhanced chemiluminescence (ECL) detection reagent was added, and the results were obtained on a molecular imager. The dual luciferase reporting system was used to further verify the targeted regulation of miRNA on *SALL4*, and the identified miRNA was denoted as miRNA-S.

Cell invasion ability was measured by Transwell methods

Tca813 cells were transfected with miRNA (mirNA-S) precursor vector, and the effects of SALL4 regulation related miRNA on invasion and migration of TCA813 cells were detected. The cells were placed in serum-free culture base and the cell concentration was adjusted to 2×10^8 cells/L for later use. After routine digestion and centrifugation, the cell density was adjusted to 2×10^8 cells/L, and 200 μL of cell suspension was gently added into Transwell chamber, then 600 μL of 10.0% serum DMEM medium was added. After the cells settled into the bottom membrane of the chamber, the whole cell was placed in an incubator for continuous culture for 12 h.

Immunohistochemistry and cell immunofluorescence assay

Tissue sections were prepared and IHC staining was performed using SP method: xylene was dewaxed for 10 min ×2 and gradient ethanol was hydrated. The slices were immersed in 3% hydrogen peroxide, protected from light for 10 min, washed with PBS 3 times, placed in plastic box of sodium citrate-EDTA antigen repair solution, repaired in microwave oven for 21 min (7 min, 14 min in medium heat), and cooled to room temperature. Wash the tablets with PBS twice, wipe the surrounding water, drop the primary antibody, and incubate the tablets overnight at 4 ℃ in a wet box, wash the tablets with PBS twice. Wipe the surrounding

water, drop the secondary antibody, and wash the tablets with PBS twice in the wet box at room temperature for 30 min. Wipe the surrounding water, drop DAB chromogenic agent, observe the staining effect under the microscope 3–5 min later, and the reaction is terminated by tap water. Wipe the surrounding water, drop hematoxylin to dye the nucleus for 5 min, tap water to stop the reaction. The film was read under optical microscope, the staining intensity was recorded, and the film was sealed. PBS buffer was used as negative control instead of primary antibody.

Statistical analysis

All data in this study were statistically collated and analyzed with the software SPSS 23.0 (IBM Corp., Armonk, NY, USA). Data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and subjected to t-test. A P value <0.05 was considered statistically significant.

Results

SALL4 mRNA expression

Tongue squamous cell carcinoma (tongue squamous carcinoma) is the most common oral and maxillofacial malignant tumor, it happened not only seriously affect the patients quality of life, but also directly affect the patient's life, how to improve the cure rate of tongue squamous carcinoma and to improve the patient's quality of life and prolong survival in patients with is that we have been exploring problems. The results showed that SALL4 mRNA expression levels in the upregulated group were higher than those in the downregulated and control groups, and the difference between the upregulated and downregulated groups was statistically significant (P<0.05) (*Table 1*).

Table 1 SALL4 mRNA expression in Tca8113 cells (mean ± SD)

Groups	SALL4
Up-regulated group	4.65±0.55
Down-regulated group	2.20±0.24
Control group	3.25±0.44

SALL4, spalt-like transcription factor 4.

SALL4 protein expression

The results showed that there was no significant difference in the expression level of SALL4 protein in Tca8113 cells of the upregulated group and downregulated groups (P>0.05), but both of them were higher than those of the control group, with statistical significance (P<0.05) (*Figure 1*).

Identification of the miRNAs related to SALL4 regulation

The results showed that the dual-luciferase reporter system further validated the targeted regulation of SALL4 by miRNA, and the identified miRNA was recorded as miRNA-S (*Figure 2*).

Cell invasion capacity

The results showed that there was no significant difference in the number of migration and invasion cells between the upregulated and downregulated Tca8113 groups

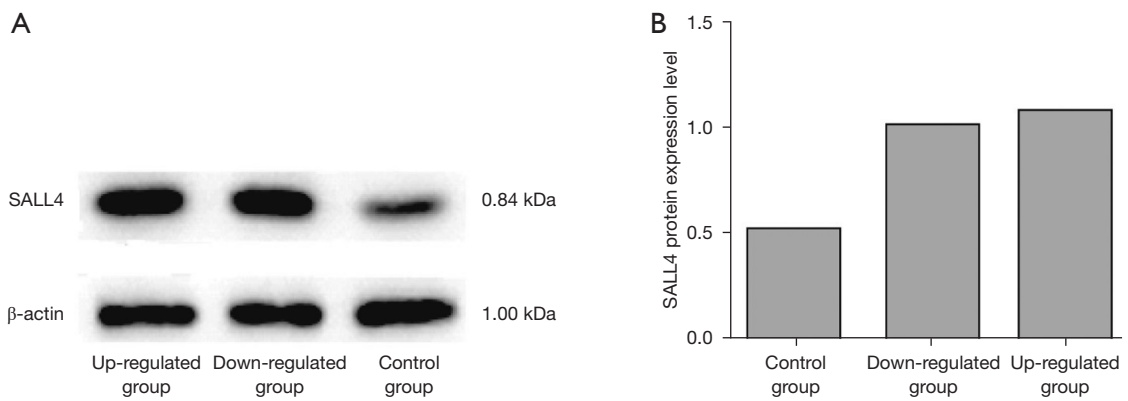

Figure 1 SALL4 protein expression in Tca8113 cells. SALL4, spalt-like transcription factor 4.

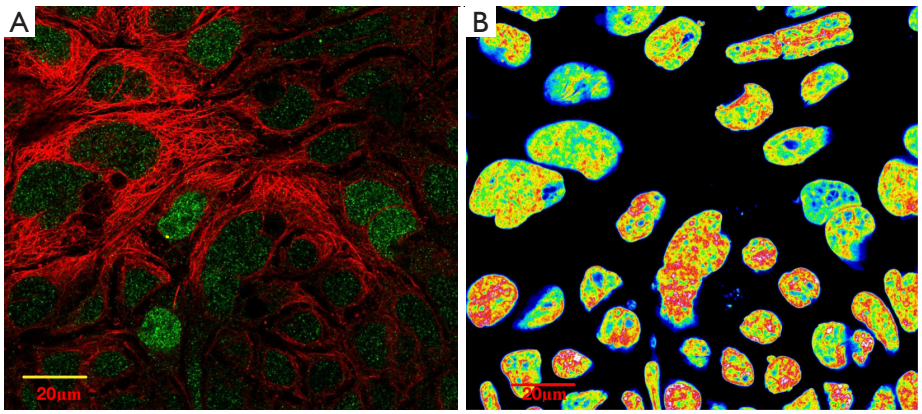

Figure 2 Immunofluorescence assay of SALL4 cells. SALL4, spalt-like transcription factor 4.

Table 2 Comparison of Tca8113 cell migration and invasion numbers (mean ± SD)

Groups	Migrating cells (%)	Invasive cells (%)
Upregulated group	10.45±1.15	14.25±0.67
Downregulated group	10.49±1.25	14.78±0.95
Control group	15.75±1.22	21.78±0.99

after intervention (P>0.05). However, they were all less than those in the control group, and the differences were statistically significant (P<0.05) (*Table 2*).

Discussion

In clinical practice, OSCC is a common malignant tumor, and with the change of people's lifestyle, the incidence of disease is on the rise, affecting the health and life of patients. Clinical studies (22,23) have shown that the occurrence and development of OSCC is a multi-factor process, which is generally believed to be related to individual genes, unfavorable living habits, tumor prevention and screening, as well as the imbalance between primary cancer and tumor suppressor genes, resulting in gene mutation or inactivation, and leading to a high recurrence rate of malignant tumors. Previously, OSCC was mainly treated with surgical resection, which can remove the focal tissue and delay the development of the disease. However, the long-term prognosis of patients is poor, and the postoperative recurrence rate is high (24). In humans, *SALL4* is a common gene which can motivate to the maturation of tissues and organs, and its expression level shows a downward trend with this maturation. However, the expression of *SALL4* gene is higher in patients with malignant tumors, which can directly participate in the occurrence and development of tumors (25). In this study, the expression level of *SALL4* mRNA in Tca8113 cells in the upregulated group was higher than that in the downregulated and control groups (P<0.05), while the expression level of *SALL4* mRNA in the control group was lower than that in downregulated group (P<0.05) (26). These results indicate that *SALL4* is highly expressed in OSCC patients and can directly participate in the occurrence and development of the disease (27).

Mutations of SALL4 are often involved in autosomal dominant diseases with multiple organ defects. Researchers outside of China have shown that *SALL4* gene is mostly highly expressed in patients with leukemia, gastric cancer, colorectal cancer, endometrial cancer, colorectal cancer, and liver cancer. Meanwhile, in the normal human blood system, *SALL4* was found to be highly expressed in CD34-positive hematopoietic stem/progenitor cells (28). As hematopoietic cells matured, the expression level of *SALL4* gene was abnormal. To further determine the role of *SALL4*-regulated miRNAs in OSCC, the dual luciferase reporting system was used in this study to further verify the targeted regulation of miRNA on SALL4, and the identified miRNA was recorded as miRNA-S. There was no significant difference in the number of migration and invasion cells in the upregulated or downregulated groups (P>0.05). The number of migration and invasion cells in both upregulated and downregulated groups were lower than that in the control group (P<0.05), suggesting that miRNA-S expression is the main expression of *SALL4*-regulated miRNAs in OSCC, which can inhibit the proliferation and growth of oral squamous cells, thus delaying the progression of disease. Pathological grading reflects the differentiation

degree of tumor cells proliferation, also reflects the degree of malignant tumor, more studies (29,30) have shown that the pathological classification, associated with the prognosis of patients with patients in the majority with high, middle differentiation, we high differentiation can be divided into a group, medium and low grade into a set of statistical analysis, found statistically significant differences in both.

The lower the pathological grade is, the worse the prognosis is. Because the lower the pathological differentiation, the faster the tumor cell proliferation, the higher the degree of malignant tumor, infiltrating range is wide, the higher the recurrence, prognosis is poorer, so it also guide us in the clinical treatment of low differentiated squamous carcinoma of the tongue to consider when appropriately expand the scope of surgical resection, adjuvant radiation and chemotherapy as far as possible with preoperative and postoperative comprehensive therapy, such as these measures may improve the prognosis of patients with tongue squamous cell carcinoma.

In conclusion, *SALL4* regulation-related miRNAs are lowly expressed in OSCC cells and can inhibit the invasion and metastasis of tumor cells. Therefore, *SALL4* is expected to be a new target for OSCC therapy.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-2974/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-2974/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-2974/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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