NF-κB-Mediated IncRNA AC007271.3 as A ceRNA Promotes Carcinogenesis of Oral Squamous Cell Carcinoma by Regulating Slug

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Research

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Abstract

**Background**: Oral squamous cell carcinoma (OSCC) is the most common oral cancer. Our previous studies confirmed that dysregulation function of long non-coding RNA (lncRNA) AC007271.3 was associated with a poor prognosis and overexpression of AC007271.3 promoted cell proliferation, migration, invasion and inhibited cell apoptosis in vitro, and promoted tumor growth in vivo. However, the underlying mechanisms of AC007271.3 dysregulation remained obscure.

**Methods**: Bioinformatics databases were used to predicted the potential down-stream targeted of AC007271.3 and verified by dual luciferase reporter assay. Core promoter region of AC007271.3 was identified by luciferase activity assay and the potential transcription factor on it was verified by ChIP assay. Western blot and qRT-PCR were performed to detect the protein and messenger RNA (mRNA) levels, respectively. Animal experiments confirmed the metastatic ability in vivo.

**Results**: AC007271.3 functioned as competing endogenous RNA (ceRNA) by binding to miR-125b-2-3p and upregulated the expression of Slug, which is a direct target of miR-125b-2-3p. AC007271.3 enhanced the expression of Slug and inhibited the expression of E-cadherin to promote the migration and invasion in OSCC cells. The expression of AC007271.3 was promoted by canonical nuclear factor-κB (NF-κB) pathway.

**Conclusion**: Our study showed that the classical NF-κB pathway-activated AC007271.3 regulates EMT by miR-125b-2-3p / Slug / E-cadherin axis to promote the development of OSCC, implicating it as a novel potential target for therapeutic intervention in this disease.

Background

OSCC is the most common type of oral cancer\(^1\). Tobacco, alcohol, betel quid and HPV infection are the major risk factors of OSCC\(^2\). Despite recent remarkable advances in the treatment of OSCC in surgery, chemotherapy, radiotherapy and targeting therapy, the 5-year survival rate is still less than 60% due to tumor recurrence and metastatic\(^3,\,4\). Therefore, understanding the detailed mechanisms of OSCC tumorigenesis and development will facilitate establishment of new effective therapeutic alternatives in order to improve the curative effect and life quality.

LncRNAs are a group of genes which longer than 200 nucleotides. They can be transcribed into mRNA but lack the ability of protein coding, which makes them considered as “functionless genes” in the past\(^5\). Over the past decade, numerous studies have shown that LncRNAs actually play important roles in human physiology and various diseases, including different kinds of cancer\(^6,\,7\). LncRNAs can functions as tumor suppressors or oncogenes, transcriptional regulation, histone modification elements, splicing and so on, which would promote or inhibit the OSCC progression\(^8\). For examples, Ding L et al identified that LncRNA-CAF, which reprograms normal fibroblasts to stromal carcinoma-related fibroblasts, promoted
Yang Y et al demonstrated that IncRNA CASC9 promoted OSCC progression by enhancing cell proliferation and suppressing autophagy-mediated cell apoptosis\textsuperscript{[9]}. AC007271.3 is a 382-nucleotides-long IncRNA which located on chromosome 2. Our previous study had identified that AC007271.3 was significantly up-regulated in OSCC than in paired adjacent normal mucosa by microarray. Higher expression of AC007271.3 in serum was related with clinical stage and poor prognosis\textsuperscript{[10]}. Further study showed that AC007271.3 mainly enriched in the cytoplasm. Overexpression of AC007271.3 promoted OSCC cells proliferation, migration, invasion and inhibited cell apoptosis in vitro, and promoted tumor growth in vivo\textsuperscript{[11]}. However, the underlying mechanisms of AC007271.3 promoting OSCC carcinogenesis remain to be excavated.

MicroRNAs(miRNAs) are a group of non-coding RNA with a length of about 22 nucleotides, which can direct the RNA-induced silencing complex to suppress the translation, or degrade target mRNA\textsuperscript{[12]}. Accumulating evidence had proved that miRNAs play a unignorable role in cancers and several miRNAs has been researched as therapeutic molecular\textsuperscript{[13]}. Recent studies indicated that IncRNAs which located in cytoplasm may play as ceRNAs of miRNAs\textsuperscript{[14]}. This phenomenon was widely discovered in various types of cancer\textsuperscript{[16,17]} and similar mechanisms also reported in OSCC. For example, IncRNA UCA1 promotes proliferation and cisplatin resistance by suppressing miR-184\textsuperscript{[18]}, and IncRNA H1 acted as a ceRNA of miR-138 to promote cell proliferation and invasion\textsuperscript{[19]}. According to the cytoplasmic location of AC007271.3, we speculated that it could play as a ceRNA of miRNAs. Further investigation verified that AC007271.3 regulated Slug by sponging miR-125b-2-3p and promoted the migration and invasion in OSCC cell lines. What's more, classical NF-κB pathway was found to activate the expression of AC007271.3. Our findings provided a novel insight into the mechanisms of AC007271.3 in promoting OSCC development, which suggested AC007271.3 could be a possible target for OSCC therapy.

Methods

**Ethics statement and tissue samples**

The OSCC tumor tissues and matched adjacent normal tissues (ANTs) in this study were collected from 82 patients with OSCC under surgery at the Department of Oral and Maxillofacial Surgery, Nanfang Hospital, Guangzhou, Guangdong Province after obtaining informed consent. The diagnosis was pathologically confirmed by the Department of Pathology, Nanfang Hospital. ANTs located at least 1.5 cm from the edge of the tumor were defined as a normal control. This study was approved by Research Scientific Ethics Committee of NanFang Hospital (NFEC-2018-027).

**Cell culture**
The human tongue squamous cell lines (SCC9, SCC15, SCC25), normal human oral keratinocyte cell line (HOK) and human embryonic kidney cell line (HEK 293T) were obtained from the Institute of Antibody Engineering, Southern Medical University (Guangzhou, China). All cells were cultured in DMEM culture medium (Gibco, NY, USA) with 10% Fetal bovine serum (ExCell Bio, Shanghai, China) and 100U/ml Penicillin-Streptomycin (Invitrogen, CA, USA) at 37°C in a humidified 5% CO₂.

**Expression plasmid, RNA oligonucleotides and transfection**

The AC007271.3 overexpression vector pcDNA3.1(+)–AC007271.3 and its corresponding control vector pcDNA3.1(+), specific siRNAs targeting AC007271.3 (si-AC007271.3), Slug(si-Slug 1, si-Slug 2, si-Slug 3) and the scramble negative control siRNA (si-AC007271.3 NC, si-Slug NC) were obtained from Sangon Biotech Corp., Ltd. (Shanghai, China). The miR-125b-2-3p inhibitor, miR-125b-2-3p micmic and the negative control (miR-125b-2-3p micmic NC, miR-125b-2-3p inhibitor NC) were purchased from RiboBio Corp, Ltd. (Guangzhou, China). Transfections were performed with Lipofectamine 3000 reagent (Invitrogen, CA, USA) following the manufacturer’s protocols.

**RNA extraction and Quantitative real-time polymerase chain reaction (qRT-PCR)**

Total cellular RNA from the cells was extracted by RNA isolater Total RNA Extraction Reagent (Vazyme, Nanjing, China) following the manufacturer’s instructions. HiScript Q RT SuperMix for qPCR®+g DNA wiper® (Vazyme, Nanjing, China) and miRNA 1st Strand cDNA Synthesis Kit (by stem-loop) (Vazyme, Nanjing, China) were used to generate cDNA. With ChamQ® SYBR® qPCR Master Mix (Vazyme, Nanjing, China), qRT-PCR was performed on the Real-Time PCR detection system (Applied Biosystems, CA, USA). All expression levels were normalized against the GAPDH or U6 (for miRNA) mRNA level. The primer sequences are listed in Supplemental Data 3.

**RNA pull-down assay**

Purified RNAs were biotin-labeled using Pierce RNA 3’End Desthiobiotinyltination Kit (Thermo Fisher Scientific, CA, USA). Biotin-labeled wild-type miR-125b-2-3p (miR-125b-2-3p-Bio), Biotin-labeled mutant-type miR-125b-2-3p (miR-125b-2-3p-Bio-MUT) and negative control (NC-Bio) were incubated with SCC9 cell lysates over night at 4°C. Magnetic beads were added to each mixture for 1 hour at room temperature. The eluted RNAs were detected by qRT-PCR.

**Western blot and Anti-body**

After 72-hours transfection with RNA oligonucleotides or plasmid, OSCC cells were lysed with RIPA buffer. Cell lysates were separated on 10% SDS–polyacrylamide gel (16mA,1.5h) and then transferred to polyvinylidene fluoride membrane (PVDF) (100V,1.5h) (Bio-Rad Laboratories). After blocking (5% non-fat dry milk in PBS Tween) for 2.5h at room temperature, the primary antibodies were used for incubation at 4°C overnight and the secondary antibodies were incubated one hour at room temperature. ECL reagent (Millipore, Bedford, MA) was used for chemiluminescence. Primary antibodies were listed as follow:
Rabbit anti-Slug (1:1000, CST, MA, USA, #9585), Rabbit anti-α-tubulin (1:1000, Abclonal, Wuhan, China, #AC007), Rabbit anti-N-cadherin (1:1000, Abclonal, Wuhan, China, #A3045), Rabbit anti-E-cadherin (1:1000, Abclonal, Wuhan, China, #A11509), Rabbit anti-Vimentin (1:2000, Proteintech, Chicago, USA, #10366-1-AP), Rabbit anti-NFKB1 (1:1000, CST, MA, USA, #13586), Rabbit anti-phospho-NFKB1-S337 (1:1000, Abclonal, Wuhan, China, #AP0125), Rabbit anti-p65 (1:1000, CST, MA, USA, #8242), Rabbit anti-Phospho-p65-Ser536 (1:1000, CST, MA, USA, #3033), Rabbit anti-Histone H3 (1:2000, CST, MA, USA, #4499).

**Cell migration and matrigel invasion assay**

OSCC cells transfected with RNA oligonucleotides or plasmid for 48h were digested and resuspended in DMEM culture. 5×10^4 cells were seeded into the upper chambers of 8-μm transwell inserts (Corning, NY, USA), and 500μl DMEM containing 20% FBS was added to the lower chamber. After 36 hours incubation at 37 °C, the membranes were fixed with methanol and stained with 0.1% crystal violet. Removed the cells on the top of the membranes and then counted the number of cells attached to the membranes under an inverted microscope. For invasion assay, matrigel (Corning, NY, USA) was diluted with DMEM (at 1:10) and covered the top membranes before cells seeding. Other steps were the same as migration assay.

**Dual luciferase reporter assay**

PmiRGLO Dual Luciferase miRNA Target Expression Vector (Promega, WI, USA) was used to construct the wild type plasmids (AC007271.3-WT, Slug-WT) which containing the potential binding sites. The mutant type plasmids (AC007271.3-MUT, Slug-MUT) were obtained from the wild type plasmid by replacing the targeted sequence (Replaced ACTTGTG with TCATCTC for AC007271.3-MUT, replaced ACTTGTGA with ACTGTCGA for Slug-MUT). All vectors were constructed by TsingKe Biological Technology Corp, Ltd. (Beijing, China). AC007271.3 WT (MUT) or Slug WT (MUT) and miR-125b-2-3p mimics (miR-125b-2-3p micmic NC) were co-transfected into HEK 293T cells. The Luc-Pair Dual-Luciferase Assay Kit 2.0 (GeneCopoeia, MD, USA) was used to detect the firefly and renilla luciferase activity. Firefly/renilla luciferase activity ratio represents the relative luciferase activities.

**Hematoxylin & Eosin (H&E) and Immunohistochemical (IHC) staining**

For histological examination, tissues were fixed with 4% paraformaldehyde at room temperature for 24 hours and washed with 70% alcohol. All tissues were embedded in paraffin and cut into sections (4 μm thick). Hematoxylin and eosin were used for H&E staining. For IHC staining, after dewaxing, washing, rehydration, antigen retrieval and endogenous peroxidase blocking following the manufacturer's protocols. Antibody Rabbit anti-Slug (1:50, Abclonal, Wuhan, China,#A1057) was used to incubate at 4°C overnight. Secondary biotinylated conjugated goat anti-rabbit antibody was incubated at room temperature for 30mins. DAB (3,3'-diaminobenzidine) were used as chromogens and hematoxylin used as counterstains. Images were acquired under the light microscope (Leica, DM 2500, Wetzlar, Germany). The comparison of staining results between tumor tissues and ATNs was performed by two independent pathologists who were blinded to the clinical information of patients. The scoring criteria of staining were...
as follow, first was staining intensity: 0-none staining, 1-light yellow, 2-deep yellow, or 3-brown; and the second criterion was staining cells proportion: 0 (<5%), 1(5–25%), 2 (25–50%), 3 (51–75%) or 4 (>75%). The product of the two scores was considered as the final score. The final scores were divided into two levels: 0–5 (Slug low expression) and more than 5 (Slug high expression).

Animal experiment

Lentivirus-NC and lentivirus-AC007271.3 were purchased from Obio Technology Corp., Ltd. (Shanghai, China) and transfected into SCC9 according to the operating instructions. Puromycin was used to select the AC007271.3 stably expressed cells (SCC9-AC007271.3) and its control cells (SCC9-NC). Five-week-old female BALB/c nude mice were randomly divided into three groups (blank, SCC9-AC007271.3 and SCC9-NC). Approximately 1 × 10^6 SCC9-AC007271.3 cells or SCC9-NC cells were resuspended in 500 μL of PBS and then injected via tail vein. Simultaneously, injection of 500 μL of PBS without cells suspension was regarded as blank group. After 8 weeks, all mice were sacrificed, and the visible lung metastases were counted. Lung metastases were excised for HE or IHC staining. The expression of AC007271.3 and miR-125b-2-3p were detected by qRT-PCR and the expression of Slug and EMT markers were inspected by western blot. This animal experiment was approved by Southern Medical University Experimental Ethics Committee (L2018199), and all BALB/c nude mice were purchased from the Animal Care Unit of Southern Medical University.

AC007271.3 Promoter Region Cloning

The sequences of the 2000bp before the 5'UTR of AC007271.3 were regarded as promoter region and obtained from Ensemble database (http://asia.ensembl.org/index.html). According to the sequences, we designed several primers with specific primers containing restriction enzyme protection sites of KpnI and Bgl II, respectively, for amplification the fragments (-1998/-2, -1508/-2, -1000/-2, -519/-2). Genomic DNA was used as a template to amplify by KOD-Plus-Neo (TOYOBO, Osaka, Japan) amplification enzyme. The reaction conditions were set following the instruction. 1% agarose gel electrophoresis was performed to select the bands in correct size and confirmed through sequencing (Sangon, Shanghai, China). The pGL3-Basic vector and correct fragments were digested with KpnI (TakaraBio, Dalian, China) and Bgl II (TakaraBio, Dalian, China) restriction enzymes. DNA ligation kit ver.2.1 (TakaraBio, Dalian, China) was used to connect the fragments and pGL3-Basic vector. DH5α competent cells were used for transformation. After the monoclonal colony sequence confirmation, the plasmids were extracted by using HiPure Plasmid EF Mini Kit (Magen, Guangzhou, China) for identification of core promoter of AC007271.3 by Dual luciferase reporter assay.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay were carried out by Magna ChIP™ A / G Kits (Merck, Darmstadt, German) according to the manufacturer's protocols. Briefly, SCC9 cells were crosslinked with 1% formaldehyde, glycine quenching and lysed. The nuclear DNA was fragmented by sonication. NFkB1 (p50) specific antibody (1:50, CST, MA, USA, #13586) was used for immunoprecipitation of cross-linked protein / DNA. Protein / DNA
complexes were eluted and reverse cross-linked into free DNA. Purified DNA was used for qRT-PCR analysis to detect the enrich fragments.

**Bioinformatics analysis**

Coding Potential Calculator ([http://cpc.cbi.pku.edu.cn/](http://cpc.cbi.pku.edu.cn/)) were performed to predict the protein coding ability of AC007271.3. LncBase Predicted v.2 database ([http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=Incbasev2%2Findex-predicted](http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=Incbasev2%2Findex-predicted)) were used to predict the potential miRNAs regulated by AC007271.3. TargetScan ([http://www.targetscan.org/](http://www.targetscan.org/)) were carried out to predict the target gene’s mRNA of miRNAs. The transcriptome profiling datum of OSCC were download from the The Cancer Genome Atlas (TCGA) database([https://portal.gdc.cancer.gov/](https://portal.gdc.cancer.gov/)) through GDC Data Transfer Tool. The Slug mRNA sequence data and relevant clinical information of 319 cases of OSCC and 32 cases of cancer adjacent normal tissues were extracted from transcriptome profiling by R software (Version 3.6.1). The miR-125b-2-3p expression analysis results of OSCC and the GEO accession number (GSE45238) were obtained from dbDEMC 2.0 ([https://www.picb.ac.cn/dbDEMC/](https://www.picb.ac.cn/dbDEMC/)). The miRNA sequence data of GSE45238 were downloaded from Gene Expression Omnibus (GEO) database ([https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45238](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE45238)) and extracted the expression data of miR-125b-2-3p. Gene-regulation online website ([http://gene-regulation.com/pub/programs.html](http://gene-regulation.com/pub/programs.html)) and JASPAR database ([http://jaspar.genereg.net/](http://jaspar.genereg.net/)) were used for transcription factor prediction.

**Statistical analysis**

All experimental assays were performed in triplicate. All data were presented as mean ± standard deviation (SD) of triplicate replicates and analyzed by SPSS statistics 20.0 (Chicago, USA). All statistical charts were manufactured in Graphpad Prism 7.0 (CA, USA). Student’s t-test was performed to compare the differences of two groups. The correlation between miR-125b-2-3p (or Slug) and clinicopathological features was analyzed by using Fisher’s exact tests. Pearson's correlation coefficient analysis was used to analyze the correlation between AC007271.3 and miR-125b-2-3p. Kaplan–Meier (K–M) curve was applied for detecting the comparison of overall survival in two groups.

**Results**

1. **MiR-125b-2-3p was a target of lncRNA AC007271.3.**

Our previous study showed that AC007271.3 could promote the proliferation, migration and invasion in OSCC [12]. To gain insight into the molecular mechanisms of AC007271.3 involved in OSCC tumorigenesis, we firstly predict the protein coding potential of AC007271.3 by using Coding Potential Calculator. As exhibited in Figure1 A, AC007271.3 had no apparent protein-coding ability. Cumulative evidence revealed that lncRNA located in the cytoplasm is vital in physiopathological processes partially by sponging miRNAs. Our previous study confirmed that AC007271.3 mainly located in cytoplasm [12], indicating that AC007271.3 may regulate target gene expression by functioning as a ceRNA of miRNAs. According to online platform LncBase Predicted v.2 database, three miRNAs (miR-4801, miR-1301-3p,
miR-125b-2-3p) which contained the complementary sequences with AC007271.3 were predicted. To verify which miRNA or miRNAs were the direct target of AC007271.3, AC007271.3 overexpression plasmid pcDNA3.1(+)-AC007271.3 was transfected into two OSCC cell lines (Figure1 B). The expression levels of three miRNAs were detected by qRT-PCR and the results showed that AC007271.3 upregulation could lead to the decrease of miR-125b-2-3p and inconsistent expression changes of miR-4801 and miR-1301-3p in SCC9 and SCC15 (Figure1 C). Furthermore, overexpression of miR-125b-2-3p (Figure1 D) resulted in the decrease of AC007271.3 (Figure1 E). Based on the complementary binding sites between AC007271.3 and miR-125b-2-3p, Dual-Luciferase reporter vectors which containing the wild or mutant binding sites were constructed and co-transfected with miR-125b-2-3p micmic or miR-125b-2-3p micmic NC in HEK-293T cells. A significant decrease of luciferase activity was observed in the AC007271.3 wild-type's group but not in the mutant type's group (Figure1 F). RNA pull-down assay exhibited that higher enrichment of AC007271.3 was observed in biotinylated miR-125b-2-3p probe (miR-125b-2-3p-Bio) than in control probe (NC-bio) or biotinylated miR-125b-2-3p mutant probe (miR-125b-2-3p-Bio-MUT) (Figure1 G).

2. **Overexpression of miR-125b-2-3p inhibited migration and invasion in OSCC.**

To investigate the roles of miR-125b-2-3p in OSCC carcinogenesis, firstly, the relative expression levels of miR-125b-2-3p in SCC9 and SCC15 cells were detected by qRT-PCR. The results showed that the expression levels of miR-125b-2-3p were significantly lower in OSCC cells than in HOK cells (Figure2 A). What’s more, compared to the matched adjacent normal tissues, the expression levels of miR-125b-2-3p were down-regulated in 82 OSCC tissues (Figure2 B). Similar analysis result was also acquired from GEO database (Figure2 C). We further investigated the association between miR-125b-2-3p expression and clinicopathological features of 82 OSCC patients. The results indicated that the expression level of miR-125b-2-3p was negatively correlated with TNM classification (p=0.0429) and lymph node metastasis (p=0.0259) in OSCC patients (Supplemental Table S1).

Considered that miR-125b-2-3p was down-regulated in OSCC tissues and cells, we next investigated the effects of miR-125b-2-3p overexpression on OSCC cell phenotypes. The results showed that miR-125b-2-3p mimic inhibited the ability of migration and invasion in SCC9 and SCC15 (Figure2 D), whereas miR-125b-2-3p inhibitor (Figure2 E) displayed the opposite results (Figure2 F).

3. **Slug acted as a target gene of miR-125b-2-3p.**

Plenty of evidence showed that miRNAs could target the 3’ untranslated region (UTR) of genes to suppress their expression. To investigate the molecular mechanism of miR-125b-2-3p suppressing the migration and invasion in OSCC cells, we predicted the target gene of miR-125b-2-3p based on the online TargetScan V7.2 software. Slug was selected as a candidate gene for its high binding score and 8mers seed sequences (Supplemental Data 1). Dual luciferase reporter assay results uncovered that obviously reduced luciferase activity was observed in Slug-wild type, while no remarkable change of luciferase activity in Slug-mutation type (Figure3 A). Furthermore, overexpression of miR-125b-2-3p decreased the protein level of Slug, while the result was just the opposite after knocking down miR-125b-2-3p (Figure3 E).
B). However, Slug mRNA had no remarkable change no matter whether miR-125b-2-3p was over-expressed or low-expressed (Supplemental Data 2).

4. **Slug was overexpressed in OSCC and silencing Slug inhibited migration and invasion in OSCC cells.**

Slug is an epithelial-mesenchymal transition (EMT) related transcription factor which could be involved in the invasion and distant metastasis of various tumor cells including OSCC\(^{[20, 21]}\). To verify the potential biological functions of Slug in OSCC, Slug expression profiles and corresponding clinical data of 319 OSCC patients and 32 normal controls were downloaded from TCGA database. Bioinformatics analysis revealed that Slug expression was remarkably up-regulated (p<0.0001) (Figure3 C) and higher expression of Slug meant poor survival and prognosis (p=0.02847) (Figure3 D). To further validate these results, the Slug expression was detected in OSCC tumor tissues and matched ANTs with immunohistochemistry. Compared to the ANTs, Slug expression was significantly higher in OSCC tumor tissues (Figure3 E). Clinicopathological characteristics analysis showed that high expression of Slug was positively correlated with TNM classification\(p=0.0301\), lymph node metastasis\(p=0.0306\) and differentiation in OSCC patients\(p=0.0465\) (Supplemental Table S2). Furthermore, we investigated the expression of Slug, β-catenin and some EMT-related markers (E-cadherin, N-cadherin and Vimentin) after knocking down Slug by specific siRNAs (si-Slug 1, si-Slug 2, si-Slug 3) against Slug transcript in OSCC cells. Western blot results showed that E-cadherin was significantly upregulated while N-cadherin, Vimentin, β-catenin had no obvious change (Figure3 F). Finally, transwell analysis indicated that the deletion of Slug could remarkably inhibited the ability of migration and invasion in OSCC cells (Figure3 G).

5. **AC007271.3 promoted migration and invasion via miR-125b-2-3p/Slug axis in OSCC cells.**

The biological function and molecular role of IncRNAs is closely associated with its subcellular localization. We previously identified that AC007271.3 was predominantly located in the cell cytoplasm by using RNA-FISH assay, which indicated that AC007271.3 might function as a ceRNA of miR-125b-2-3p and regulate Slug. To further determine the relationship among AC007271.3, miR-125b-2-3p and Slug, firstly, Pearson's correlation analysis was performed to uncover the association between AC007271.3 and miR-125b-2-3p expression in 82 OSCC tissues. The results indicated that the expression of miR-125b-2-3p was inversely correlated with AC007271.3 (Figure4 A). Then, the upregulation of Slug and downregulation of E-cadherin were observed when overexpressing AC007271.3, while knocking down AC007271.3 resulted in the opposite results (Figure4 B). In addition, the up-regulated Slug and down-regulated E-cadherin caused by transfecting pcDNA3.1(+) AC007271.3 was reversed when co-transfecting with miR-125b-2-3p mimics (Figure4 C). Simultaneously, migration and invasion experiments revealed that miR-125b-2-3p mimics and si-Slug could reversed the effects resulted from AC007271.3 overexpression (Figure4 D-E).

6. **Upregulation of AC007271.3 promoted OSCC metastasis in vivo.**

In our previous study\(\text{we had identified that the upregulation of AC007271.3 promoted OSCC tumor growth in vivo. To evaluate the effect of AC007271.3 on OSCC metastasis in vivo, AC007271.3 stably over-expressed SCC9 (SCC9-AC007271.3) cells and their control cells (SCC9-NC) were independently}
injected into the tail vein of BALB/c\(\text{nu/nu}\) mice to establish a pulmonary metastasis model. 8 weeks later, the mice were sacrificed and lung tissues were photographed. We could clearly see more pulmonary metastatic nodules in SCC9-AC007271.3 group than in SCC9-NC group, which suggesting that AC007271.3 could significantly promote OSCC metastasis to lung tissues (Figure5 A-B). QRT-PCR analysis showed that the expression of AC007271.3 in pulmonary metastases was dramatically increased, while miR-125b-2-3p’s RNA level was significantly decreased in the SCC9-AC007271.3 group in contrast to that in the SCC9-NC group (Figure5 C). Furthermore, H&E staining of lung sections indicated that overexpression of AC007271.3 increased the number and size of pulmonary metastases. The results of IHC staining and western blot also revealed a significant upregulation of Slug in pulmonary metastases of SCC9-AC007271.3 group compared with that in SCC9-NC group (Figure5 D-E). These results indicated that AC007271.3 contributed to OSCC metastasis in vivo.

7. The expression of AC007271.3 was promoted by canonical NF-\(\kappa\)B pathway.

In order to explore the upstream regulation mechanism of AC007271.3, we firstly consider the 2000bp before the 5'UTR of AC007271.3 to be the promoter region and obtained the promoter region sequences on Ensemble database. According to these sequences, we designed several primers (Supplemental Data 3) to constructed 4 fragment-by-fragment deletion pGL3 vectors of AC007271.3 promoter (-1998/-2, -1508/-2, -1000/-2, -519/-2). Luciferase activity assay was performed for the identification of core promoter region of AC007271.3 and the unidirectional deletion at -1000/-519 caused significant reduction in the luciferase activity (Figure6 A) so we preliminarily regarded -1000/-519 region as the core promoter of AC007271.3. Next, we predicted that NF-\(\kappa\)B may be the potential transcription factor on the core promoter of AC007271.3 by using three different programs (Match - 1.0, Patch 1.0 and AliBaba 2.1) on Gene-regulation online website. NF-\(\kappa\)B is a dimer formed by different kinds of subunits. The prediction on JASPAR database indicated that NFKB1 may be the most critical subunit (Figure6 B, Supplemental Table S3). To verify our conjecture, we designed 3 pairs of primers according to the predicted binding sites (AC007271.3-promoter Site 1: -953/-943, AC007271.3-promoter Site 2: -854/-842, AC007271.3-promoter Site 3: -581/-571) and carried out ChIP-qRT-PCR analysis, which suggested that NFKB1 enriched on the AC007271.3-promoter Site 2 (Figure6 C). NFKB1 is also known as p50, and p50/p65 is the most common type of NF-\(\kappa\)B dimer, which is activated by TNF-\(\alpha\) in classical NF-\(\kappa\)B pathway and plays a positive regulatory role during gene transcription. Therefore, we measured the mRNA level of AC007271.3, miR-125b-2-3p and Slug after TNF-\(\alpha\) (10ng/ml) treatment for the indicated times and the results showed that the mRNA level of AC007271.3 and Slug were stably activated after 0.5h and 2h, respectively. On the other hand, interestingly, the mRNA level of miR-125b-2-3p strongly increased after 0.5h but gradually decreased with the increasing time until 72h (Figure6 D). Western blot analysis result indicated that the protein level of Slug was also up-regulated after TNF-\(\alpha\) treatment for 72h (Figure6 E), and at the same time, nuclear translocation of p65 and nuclear-phosphorylation of p50 and p65 were detected (Figure6 F). These results suggested that the activation of canonical NF-\(\kappa\)B pathway could positively regulate AC007271.3 and affect the expression of miR-125b-2-3p and Slug.
Discussion

Accumulating evidence indicated that IncRNA is closely related to tumorigenesis and development. Our recent studies confirmed that aberrant AC007271.3 levels in OSCC patients were significantly associated with clinical stage, especially in early-stage disease and serum AC007271.3 levels could also discriminate between OSCC and normal controls with high sensitivity and specificity, which suggesting that AC007271.3 could be a novel circulating biomarker for the determination of OSCC\(^\text{[11]}\). Furthermore, we found that AC007271.3 could promote cell proliferation, invasion and inhibit cell apoptosis of OSCC via the Wnt/\(\beta\)-catenin signaling pathway, which might provide a novel therapeutic approach for OSCC\(^\text{[12]}\). In this study, we found that overexpression of AC007271.3 could facilitate the metastasis of OSCC cells in vivo, which further demonstrated the tumorigenesis role of AC007271.3. However, the underlying mechanism of AC007271.3 in OSCC carcinogenesis keep unclear.

Plenty of evidence has indicated that IncRNAs can regulate target gene expression by functioning as a ceRNA for miRNA\(^\text{[15,22]}\). LncRNA functions are closely associated its subcellular localization\(^\text{[23]}\). We proved that AC007271.3 was mainly located in the cytoplasm. Thus, it indicated that AC007271.3 may function as an endogenous miRNA sponge to regulate the expression of target genes. In the present study, bioinformatics analysis and luciferase reporter assays revealed that miR-125b-2-3p is a target of AC007271.3. Moreover, AC007271.3 upregulation could decrease miR-125b-2-3p expression and deletion of miR-125b-2-3p could inhibit the expression of AC007271.3, which confirmed that AC007271.3 served as a ceRNA with miR-125b-2-3p. To our knowledge, this is the first report to manifest that AC007271.3 can be regulated by miR-125b-2-3p in OSCC.

MiR-125b-2-3p is a member of human miR-125 family, which consists of three homologs (miR-125a, miR-125b-1, and miR-125b-2). The 125b-2-3p was derived from the 3' arm of the precursor miRNA(pre-miRNA) of miR-125b-2\(^\text{[24]}\). It had reported that miR-125b-2-3p was down-regulated in hepatocellular carcinoma\(^\text{[25]}\) and small cell osteosarcoma\(^\text{[26]}\) but upregulated in tumor stroma of colon cancer\(^\text{[27]}\). In OSCC, although miR-125b was found to inhibit the progression of OSCC\(^\text{[28]}\), the function of miR-125b-2-3p is still uncertain. In our study, we confirmed that the expression levels of miR-125b-2-3p were negatively correlated with TNM classification and lymph node metastasis in OSCC patients. Overexpression of miR-125b-2-3p could inhibit the migration and invasion of OSCC, while silencing of miR-125b had the opposite result. These results indicated that miR-125b-2-3p existed genetic heterogeneity in tumor. However, the underlying mechanisms of miR-125b-2-3p aberrant dysregulation in tumor are unknown. In hepatocellular carcinoma, low expression of miR-125b-2-3p could increase the expression of the target gene cyclin A2, further promoting the formation of CycA/CDK1 and then affect the G2/M phase of the cell cycle\(^\text{[25]}\).

Slug, also known as Snail Family Transcriptional Repressor 2 (Snai2), is a member of the Snail family (Snai1, Snai2, Snai3). It's an important transcription factor and plays a vital role in development and closely relates to the occurrence and development of various diseases including cancer\(^\text{[29]}\). Aberrant expression of Slug has been closely related to cancer stem cell formation, cell cycle regulation and
apoptosis as well as invasion and metastasis\textsuperscript{[21]}. As we predicted in the present study, Slug was a target gene of miR-125b-2-3p by bioinformatics analysis and then verified by luciferase reporter assays and western blot. The expression of Slug was remarkably up-regulated in OSCC tissues than in normal controls and meant unfavorable prognosis by bioinformatics analysis. We further found that Slug expression was significantly higher than in the adjacent normal tissues by immunohistochemistry and high expression of Slug was positive correlated with TNM classification, lymph node metastasis and differentiation in OSCC patients. In addition, knockdown of Slug expression remarkably impaired the ability of migration and invasion of OSCC cells. These results discovered that Slug might play an important role in OSCC carcinogenesis. In this study, we found that AC007271.3 positively regulated the expression of Slug. In the rescue experiment, over-expression of miR-125b-2-3p reversed the up-regulated Slug caused by over-expressed AC007271.3. Simultaneously, migration and invasion experiments unveiled that miR-125b-2-3p mimics and si-Slug can reversed the effects resulted from AC007271.3 overexpression. Take together, AC007271.3 could regulate Slug expression by sequestering endogenous miR-125b-2-3p.

Snai family members could directly bind to the promoter of E-cadherin by E-box region and negatively regulate its expression\textsuperscript{[30, 31]}. E-cadherin, N-cadherin and Vimentin were the frequent symbol markers for EMT\textsuperscript{[32]}, which could enhance the invasion and migration of tumors. In this work, we found that the knockdown of Slug induced no significant changes on N-cadherin, Vimentin and β-catenin except a remarkably decrease of E-cadherin, which indicated that Slug could change the epithelial-mesenchymal-transition phenotype by inhibiting the expression of E-cadherin and then promoted the migration and invasion in OSCC cells. On the other hand, the E-cadherin / catenin complex, which bind to cytoskeletal components, is an important regulator to form a mature adherent junction\textsuperscript{[33]}. The reduction in E-cadherin was related with the activating of β-catenin in colorectal cancer\textsuperscript{[34]}. Combined with our results, we speculated that Slug may increase the dissociated β-catenin which could be activated and translocated into the nucleus by reducing the expression of E-cadherin. Moreover, recent researches implied that Wnt / β-catenin pathway participated in the regulation of Slug\textsuperscript{[35, 36]}, which may form a feed-back regulation between Slug/E-cadherin/β-catenin. However, the relation among Slug, E-cadherin and β-catenin in OSCC need to be further explored.

NF-κB is a regulator of expression of the κB light chain which firstly recognized in B cells. It is a homo- or hetero-dimer formed by any two of the five subunits of RelA (p65), RelB, c-Rel, NFKB1 (p50), NFKB2 (p52) \textsuperscript{[37]}. Plenty of evidence indicated that NF-κB pathway played an important role in inflammation and cancer\textsuperscript{[38, 39]}. In the canonical NF-κB pathway, tumor necrosis factor (TNF)-α stimulation induces IKKβ phosphorylation and then phosphorylates IkBα to promote its polyubiquitination and degradation, which lead to the release of p65/p50 heterodimer. P65/p50 dimers may be phosphorylated\textsuperscript{[40]} and then translocate into nucleus and bind to the specific DNA sequences to promote the transcription of target genes\textsuperscript{[41]}. The NF-κB pathway has been fully studied in the past few decades. It was widely acknowledged that p50 / p65 dimer plays a direct role in the canonical NF-κB pathway to positively regulate the transcription of target genes\textsuperscript{[37–39, 41]}. Phosphorylation sites of p50 and p65 also regulate the
function of this dimer\textsuperscript{[40, 42]}, Previous studies showed that p50 (Ser337) site phosphorylation could enhance the DNA binding ability of p50\textsuperscript{[43]}, while p65 (Ser536) phosphorylation could enhance transactivation potential of p65\textsuperscript{[44]}. In the research, after treatment of TNF-α in SCC9, the over-expressed AC007271.3 were detected, and the p50 (Ser337) and p65 (Ser536) were significantly up-regulated in nucleus. These results suggested that the activation of canonical NF-κB pathway could positively regulate the expression of AC007271.3. In addition, recent study showed that NF-κB pathway promoted the metastasis in head and neck squamous cell carcinoma cells by stabilizing Slug\textsuperscript{[45]}. However, the underlying mechanism is not clear. Our results identified that classical NF-κB pathway probably regulated the migration and invasion of OSCC through the AC007271.3 / miR-125b-2-3p / Slug axis. To our knowledge, it’s the first time to propose this potential mechanism in OSCC.

**Conclusion**

In conclusion, our research confirmed that the classical NF-κB pathway-regulated AC007271.3 played as a ceRNA of miR-125b-2-3p to regulate Slug gene and promote the migration and invasion in OSCC cells, which suggested that AC007271.3 may be a diagnostic molecule and therapeutic target for OSCC.

**List Of Abbreviations**

ATNs
adjacent normal tissues; ceRNA:competing endogenous RNA; ChIP:chromatin immunoprecipitation; EMT:epithelial-mesenchymal transition; GEO:Gene Expression Omnibus; H&E:Hematoxylin & Eosin; IHC:Immunohistochemical; IncRNA:long non-coding RNA; IncRNA AC007271.3:long non-coding RNA AC007271.3; miRNA:MicroRNA; miR-125b-2-3p:microRNA-125b-2-3p; mRNA:Messenger RNA; NF-κB:nuclear factor-κB; OSCC:oral squamous cell carcinoma; qRT-PCR:Quantitative real-time polymerase chain reaction; Slug or Snai2:Snail Family Transcriptional Repressor 2; TCGA:The Cancer Genome Atlas; UTR:untranslated region.

**Declarations**

**Ethics approval and consent to participate:** The clinical tissues collection was approved by Research Scientific Ethics Committee of NanFang Hospital (NFEC-2018-027). The animal experiment was approved by Southern Medical University Experimental Ethics Committee (L2018199).

**Consent for publication:** Not applicable.

**Availability of data and materials:** The datasets used during the current study are available from the corresponding author on reasonable request.

**Competing interests:** The authors declare that they have no competing interests.
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**References**

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries[J]. CA Cancer J Clin. 2018;6:394–424.

2. Chi AC, Day TA, and B. W. Neville. Oral cavity and oropharyngeal squamous cell carcinoma–an update[J]. CA Cancer J Clin. 2015;5:401–21.

3. Khan T, Relitti N, Brindisi M, Magnano S, Zisterer D, Gemma S. S. Butini and G. Campiani. Autophagy modulators for the treatment of oral and esophageal squamous cell carcinomas[J]. Med Res Rev, 2019.

4. Chen IC, Chiang WF, Huang HH, Chen PF, Shen YY, and H. C. Chiang. Role of SIRT1 in regulation of epithelial-to-mesenchymal transition in oral squamous cell carcinoma metastasis[J]. Mol Cancer, 2014, 254.

5. Quinn JJ, Chang HY. Unique features of long non-coding RNA biogenesis and function[J]. Nat Rev Genet. 2016;1:47–62.

6. Schmitt AM, Chang HY. Long Noncoding RNAs in Cancer Pathways[J]. Cancer Cell. 2016;4:452–63.

7. Kopp F, Mendell JT. Functional Classification and Experimental Dissection of Long Noncoding RNAs[J]. Cell. 2018;3:393–407.

8. Zhang L, Meng X, Zhu XW, Yang DC, Chen R, Jiang Y, Xu T. Long non-coding RNAs in Oral squamous cell carcinoma: biologic function, mechanisms and clinical implications[J]. Mol Cancer. 2019;1:102.

9. Ding L, Ren J, Zhang D, Li Y, Huang X, Hu Q, Wang H, Song Y, Ni Y, Hou Y. A novel stromal IncRNA signature reprograms fibroblasts to promote the growth of oral squamous cell carcinoma via LncRNA-CAF/interleukin-33[J]. Carcinogenesis. 2018;3:397–406.

10. Yang Y, Chen D, Liu H, Yang K. Increased expression of IncRNA CASC9 promotes tumor progression by suppressing autophagy-mediated cell apoptosis via the AKT/mTOR pathway in oral squamous cell carcinoma[J]. Cell Death Dis. 2019;2:41.
11. Shao T, Huang J, Zheng Z, Wu Q, Liu T, Lv X. SCCA, TSGF, and the Long Non-Coding RNA AC007271.3 are Effective Biomarkers for Diagnosing Oral Squamous Cell Carcinoma[J]. Cell Physiol Biochem. 2018;1:26–38.

12. Shao TR, Zheng ZN, Chen YC, Wu QQ, Huang GZ, Li F. W. S. Zeng and X. Z. Lv. LncRNA AC007271.3 promotes cell proliferation, invasion, migration and inhibits cell apoptosis of OSCC via the Wnt/beta-catenin signaling pathway[J]. Life Sci, 2019, 117087.

13. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function[J]. Cell. 2004;2:281–97.

14. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases[J]. Nat Rev Drug Discovery. 2017;3:203–21.

15. Thomson DW, Dinger ME. Endogenous microRNA sponges: evidence and controversy[J]. Nat Rev Genet. 2016;5:272–83.

16. Zhang G, Li S, Lu J, Ge Y, Wang Q, Ma G, Zhao Q, Wu D, Gong W, Du M, Chu H, Wang M, Zhang A, Zhang Z. LncRNA MT1JP functions as a ceRNA in regulating FBXW7 through competitively binding to miR-92a-3p in gastric cancer[J]. Mol Cancer. 2018;1:87.

17. Li Y, Guo D, Zhao Y, Ren M, Lu G, Wang Y, Zhang J, Mi C, He S, Lu X. Long non-coding RNA SNHG5 promotes human hepatocellular carcinoma progression by regulating miR-26a-5p/GSK3beta signal pathway[J]. Cell Death Dis. 2018;9:888.

18. Fang Z, Zhao J, Xie W, Sun Q, Wang H, Qiao B. LncRNA UCA1 promotes proliferation and cisplatin resistance of oral squamous cell carcinoma by supppressing miR-184 expression[J]. Cancer Med. 2017;12:2897–908.

19. Hong Y, He H, Sui W, Zhang J, Zhang S, Yang D. Long non-coding RNA H1 promotes cell proliferation and invasion by acting as a ceRNA of miR138 and releasing EZH2 in oral squamous cell carcinoma[J]. Int J Oncol. 2018;3:901–12.

20. Joseph MJ, Dangi-Garimella S, Shields MA, Diamond ME, Sun L, Koblinski JE. and H. G. Munshi. Slug is a downstream mediator of transforming growth factor-beta1-induced matrix metalloproteinase-9 expression and invasion of oral cancer cells[J]. J Cell Biochem. 2009;3:726–36.

21. Zhou W, Gross KM, Kuperwasser C. Molecular regulation of Snai2 in development and disease[J]. J Cell Sci, 2019, 23.

22. Huang GZ, Wu QQ, Zheng ZN, Shao TR, and X. Z. Lv. Identification of Candidate Biomarkers and Analysis of Prognostic Values in Oral Squamous Cell Carcinoma[J]. Front Oncol, 2019, 1054.

23. Yao RW, Wang Y, Chen LL. Cellular functions of long noncoding RNAs[J]. Nat Cell Biol. 2019;5:542–51.

24. Sun YM, Lin KY, Chen YQ. Diverse functions of miR-125 family in different cell contexts[J]. J Hematol Oncol, 2013, 6.

25. Shi J, Ye G, Zhao G, Wang X, Ye C, Thammavong K, Xu J, Dong J. Coordinative control of G2/M phase of the cell cycle by non-coding RNAs in hepatocellular carcinoma[J]. PeerJ, 2018, e5787.
26. Xie L, Liao Y, Shen L, Hu F, Yu S, Zhou Y, Zhang Y, Yang Y, Li D, Ren M, Yuan Z, Yang Z. Identification of the miRNA-mRNA regulatory network of small cell osteosarcoma based on RNA-seq[J]. Oncotarget. 2017;26:42525–36.

27. Zhou XG, Huang XL, Liang SY, Tang SM, Wu SK, Huang TT, Mo ZN, Wang QY. Identifying miRNA and gene modules of colon cancer associated with pathological stage by weighted gene co-expression network analysis[J]. Onco Targets Ther, 2018, 2815–2830.

28. Chen YF, Wei YY, Yang CC, Liu CJ, Yeh LY, Chou CH, Chang KW, Lin SC. miR-125b suppresses oral oncogenicity by targeting the anti-oxidative gene PRXL2A[J]. Redox Biol, 2019, 101140.

29. Cobaleda C, Perez-Caro M, Vicente-Duenas C, Sanchez-Garcia I. Function of the zinc-finger transcription factor SNAI2 in cancer and development[J]. Annu Rev Genet, 2007, 41–61.

30. Hajra KM, Chen DY, and E. R. Fearon. The SLUG zinc-finger protein represses E-cadherin in breast cancer[J]. Cancer Res. 2002;6:1613–8.

31. Yang MH, Chen CL, Chau GY, Chiou SH, Su CW, Chou TY, Peng WL, Wu JC. Comprehensive analysis of the independent effect of twist and snail in promoting metastasis of hepatocellular carcinoma[J]. Hepatology. 2009;5:1464–74.

32. Nieto MA, Huang RY, Jackson RA, and J. P. Thiery Em: 2016[J] Cell. 2016;1:21–45.

33. Chen HN, Yuan K, Xie N, Wang K, Huang Z, Chen Y, Dou Q, Wu M, Nice EC, Zhou ZG, Huang C. PDLIM1 Stabilizes the E-Cadherin/beta-Catenin Complex to Prevent Epithelial-Mesenchymal Transition and Metastatic Potential of Colorectal Cancer Cells[J]. Cancer Res. 2016;5:1122–34.

34. Huels DJ, Ridgway RA, Radulescu S, Leushacke M, Campbell AD, Biswas S, Leedham S, Serra S, Chetty R, Moreaux G, Parry L, Matthews J, Song F, Hedley A, Kalna G, Ceteci F, Reed KR, Meniel VS, Maguire A, Doyle B, Soderberg O, Barker N, Watson A, Larue L, Clarke AR, and O. J. Sansom. E-cadherin can limit the transforming properties of activating beta-catenin mutations[J]. EMBO J. 2015;18:2321–33.

35. Heuberger J, Birchmeier W. Interplay of cadherin-mediated cell adhesion and canonical Wnt signaling[J]. Cold Spring Harb Perspect Biol. 2010;2:a002915.

36. Wu ZQ, Li XY, Hu CY, Ford M, Kleer CG and S. J. Weiss. Canonical Wnt signaling regulates Slug activity and links epithelial-mesenchymal transition with epigenetic Breast Cancer 1, Early Onset (BRCA1) repression[J]. Proc Natl Acad Sci U S A, 2012, 41:16654-9.

37. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling[J]. Cell. 2008;3:344–62.

38. DiDonato JA, Mercurio F, Karin M. NF-kappaB and the link between inflammation and cancer[J]. Immunol Rev. 2012;1:379–400.

39. Hoesel B, Schmid JA. The complexity of NF-kappaB signaling in inflammation and cancer[J]. Mol Cancer, 2013, 86.

40. Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation[J]. Trends Biochem Sci. 2005;1:43–52.
41. Bakkar N, Guttridge DC. NF-kappaB signaling: a tale of two pathways in skeletal myogenesis[J]. Physiol Rev. 2010;2:495–511.

42. Lu X, Yarbrough WG. Negative regulation of RelA phosphorylation: emerging players and their roles in cancer[J]. Cytokine Growth Factor Rev. 2015;1:7–13.

43. Hou S, Guan H, Ricciardi RP. Phosphorylation of serine 337 of NF-kappaB p50 is critical for DNA binding[J]. J Biol Chem. 2003;46:45994–8.

44. Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W. IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain[J]. J Biol Chem. 1999;43:30353–6.

45. Liu S, Shi L, Wang Y, Ye D, Ju H, Ma H, Yang W, Wang Y, Hu J, Deng J, Zhang Z. Stabilization of Slug by NF-kappaB is Essential for TNF-alpha -Induced Migration and Epithelial-Mesenchymal Transition in Head and Neck Squamous Cell Carcinoma Cells[J]. Cell Physiol Biochem. 2018;2:567–78.

**Figures**
AC007271.3 interacted with miR-125b-2-3p. a. The prediction of AC007271.3 protein coding ability. b. The overexpression efficiency of pcDNA3.1(+)–AC007271.3 in SCC9 and SCC15 cells. c. The expression changes of three predicted miRNAs after overexpressing AC007271.3. MiR-125b-2-3p were both significantly down-regulated in SCC9 and SCC15. d. The overexpression efficiency of miR-125b-2-3p micmic in SCC9 and SCC15 cells. e. The upregulation of miR-125b-2-3p inhibited the expression of...
AC007271.3. f. MiR-125b-2-3p overexpression reduced the relative luciferase activity in AC007271.3-WT but not in AC007271.3-MUT. g. RNA pull-down assay indicated the direct interaction between miR-125b-2-3p and AC007271.3 in SCC9. *p < 0.05, **p < 0.01, ***p<0.001.

Figure 2

MiR-125b-2-3p regulated migration and invasion in OSCC cells. a. Lower expression of miR-125b-2-3p was detected in two OSCC cells compared to HOK cells. b. Differential expression of miR-125b-2-3p in 82
pairs of OSCC clinical samples ($\Delta$CT). c. The expression data of miR-125b-2-3p in 40 pairs of OSCC in GEO database. d. Overexpressing miR-125b-2-3p inhibited the migration(left) and invasion(right) in SCC9 and SCC15. e. The inhibition efficiency of miR-125b-2-3p inhibitor in SCC9 and SCC15 cells. f. Inhibiting the expression of miR-125b-2-3p promoted the migration(left) and invasion(right) ability in SCC9 and SCC15. *p < 0.05, **p < 0.01, ***p<0.001, ****p<0.0001.

Figure 3
Slug was a target gene of miR-125b-2-3p and positively correlated with the malignancy of OSCC. a. MiR-125b-2-3p overexpression reduced the relative luciferase activity in Slug-WT. b. Overexpressing or inhibiting miR-125b-2-3p changed the expression of Slug protein in SCC9 (left) and SCC15(right). c. The expression of Slug in OSCC(n=319) and normal (n=32) tissues in TCGA database. d. Kaplan-Meier analysis were employed to estimate the relation between Slug expression and overall survival of OSCC patients. e. Representative images of Slug expression in the OSCC tumor and adjacent non-malignant tissue analyzed by immunohistochemical. f. After transfecting si-Slug in SCC9 and SCC15, the expression of Slug, E-cadherin, N-cadherin, Vimentin and β-catenin were detected by western blot. g. Si-Slug inhibited the migration(left) and invasion(right) in SCC9 and SCC15. *p < 0.05, **p < 0.01, ***p<0.001.
Figure 4

AC007271.3 promoted migration and invasion via miR-125b-2-3p / Slug in OSCC cells. a. Pearson's correlation analysis of the relationship between AC007271.3 and miR-125b-2-3p. b. Western blot analysis was performed to detect the expression of Slug and EMT-related markers after overexpressing (or knocking down) AC007271.3 in SCC9 and SCC15. c. MiR-125b-2-3p micm could reversed the changing
expression of Slug and E-cadherin caused by overexpressed AC007271.3. d-e. Effects of pcDNA3.1(+)AC007271.3, miR-125b-2-3p mimics, si-Slug on the migration (d) and invasion (e) in SCC9 and SCC15.

Figure 5

AC007271.3 promoted OSCC metastasis in vivo. a. Representative images of pulmonary metastatic models. b. Statistical analysis of colony numbers of pulmonary metastases between SCC9-AC007271.3 and SCC9-NC group. c. The expression of AC007271.3 and miR-125b-2-3p in pulmonary metastases were
detected by qRT-PCR. d. Representative images of HE and IHC staining. Higher level of Slug was detected in SCC9-AC007271.3 group than that in SCC9-NC group. e. Western blot confirmed the up-regulated of Slug and the down-regulated of E-cadherin in SCC9-AC007271.3 group. *p < 0.05, **p < 0.01, ***p<0.001.

NFkB1 was enriched on the core promoter region of AC007271.3 and canonical NF-κB pathway upregulated the expression of AC007271.3. a. After transfecting fragment-by-fragment deletion pGL3...
vectors of AC007271.3 promoter into 293T cells for 48 hours, the luciferase activity was measured and analyzed statistically. b. The binding sequence logo of NFKB1 on JASPAR database. c. Enrichment of NFKB1 was detected at the Site 2 (-854/-842) of AC007271.3 core promoter in SCC9 by ChIP-qRT-PCR analysis. d. After treating with TNF-α (10 ng/ml) for the indicated times in SCC9, the mRNA level of AC007271.3, miR-125b-2-3p and Slug was analyzed by qRT-PCR. GAPDH was used as a control. e. The western blot analysis indicated that the protein level of Slug also increased by TNF-α treatment (10 ng/ml, 72 hours). α-tubulin was employed as a control. f. The canonical NF-κB pathway related proteins in the cytoplasmic and nuclear of SCC9 which treated with or without TNF-α (10 ng/ml, 72 h) were assessed by western blot. α-tubulin and Histone H3 were employed as the positive controls for cytoplasmic and nuclear proteins, respectively. *p < 0.05, **p < 0.01, ***p<0.001.

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