Abstract. Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the oral cavity. Emerging evidence indicates that long non-coding (lnc)RNAs play a key role in the cellular processes of tumor cells, including glycolysis, growth and movement. Here, the purpose of this study was to explore the biological functions and potential mechanism of lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) in OSCC. OSCC tissues and adjacent matched paraneoplastic normal tissues were collected from 20 OSCC patients. The expression of MALAT1 and miR-101 in OSCC tissues and cell lines (HSC3, SCC9, SCC15 and SCC25) were determined by real-time-polymerase chain reaction (qPCR). Caspase-3, caspase-8 and EZH2 protein levels were determined by western blot analysis. MALAT1-mediated miRNAs were verified by bioinformatics analysis of StarBase and Luciferase reporter assay. Cell Counting Kit-8 (CCK-8) and Transwell assays were used for investigating MALAT1 effect on cell proliferation and invasion in the OSCC cells. qPCR analysis indicated that MALAT1 expression was obviously increased, and miR-101 was decreased in the OSCC tissues and cell lines. Functional studies revealed that overexpression of MALAT1 promoted OSCC cell proliferation and invasion. Further experiments revealed that miR-101 was a target of MALAT1 and that the miR-101 inhibitor abolished the effect of MALAT1 on OSCC cell proliferation and invasion. Enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) acted as a downstream effector of MALAT1 in the OSCC cells. Collectively, these findings revealed that upregulation of MALAT1 facilitated OSCC proliferation and invasion by targeting the miR-101/EZH2 axis.

Introduction

Oral squamous cell carcinoma (OSCC) is the eighth most common tumor worldwide, and accounts for 2.1% of deaths caused by all cancers (1,2). Oral cancer consists of a group of neoplasms that affect regions of the oral cavity, pharyngeal regions and salivary glands (3). Although great advances have been made in the treatment of OSCC, including surgery, chemotherapy, radiotherapy and hormonotherapy, the 5-year survival rate of OSCC remains less than 50%, mainly due to cancer metastasis to lymph nodes (4). Therefore, there is an urgently need to explore the molecular mechanisms underlying OSCC to improve therapeutic efficacy and the survival rate of OSCC patients.

Long non-coding RNAs (lncRNAs) are a class of non-coding RNAs more than 200 nucleotides in length. Increasing evidence suggests that lncRNAs are notable molecular markers involved in the regulation of gene expression and cancer progression (5,6). Dysfunction of lncRNAs are closely related to the incidence of human diseases, including tumors, degenerative neurological diseases and other major diseases that seriously endanger human health (7,8). Thus, currently lncRNAs are a ‘hot spot’ of tumor research. IncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was first discovered in non-small cell lung cancer (NSCLC), and is located on chromosome 11q13.1 which is highly conserved (9). MALAT1 was reported to be upregulated in colorectal cancer (CRC) and to promote cell proliferation, metastasis and invasion (10). However, the molecular mechanisms of MALAT1 in mediating the progression of OSCC have not been well investigated.

MicroRNAs (miRNAs/miRs) are a large family of short non-coding RNAs, consisting of 18-25 nucleotides, and have been found to negatively regulate target genes via binding to the 3'-UTR (3'-untranslated region) (11). miR-101 has been
reported to be downregulated in OSCC cells and to act as a tumor suppressor in OSCC development (12). It is worth mentioning that miR-101 was suggested to be a potential target of MALAT1 by Starbase. However, the exact role of miR-101 in MALAT-mediated OSCC progression needs further investigation.

As previously elucidated, miRNAs are involved in tumor development by targeting their mRNAs. Enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) has been verified as a direct target of miR-101 in various types of cancers, including CRC, laryngeal squamous cell carcinoma and retinoblastoma (13-15). However, whether EZH2 serves as the target of miR-101 in the modulation of OSCC progression has not yet been clarified.

Materials and methods

Tissue specimens. Twenty paired OSCC tissues and adjacent normal tissues were obtained from The Affiliated Yantai Yuhuangding Hospital of Qingdao University (Yantai, Shandong, China) between March 2015 and April 2018. All patients (age range, 35-75 years, mean 61.3 years; 16 male and 4 female) were not treated with radiotherapy or chemotherapy prior to surgery. Liquid nitrogen was used to flash-freeze all the tissue specimens, and then the collected tissues were stored at -80°C for further use. All patients signed written informed consent. This study was approved by the Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University (no. 20140506).

Cell culture. OSCC cell lines (HSC3, SCC9, SCC15 and SCC25) and primary normal human oral keratinocyte (NHOK) cells were purchased from the BeNa Culture Collection (BNCC, Shanghai, China). RPMI-1640 medium containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin were used to culture OSCC cells and then cells were incubated at 37°C with 5% CO2 atmosphere.

Cell transfection. Small interference RNA (siRNA), scrambled oligonucleotide (NC) vector, MALAT1 siRNA, MALAT1 vector, miR-101 mimic, miR-101 inhibitor, negative control (NC)-mimic, NC-inhibitor, EZH2 plasmid and NC-plasmid were designed and synthesized by GenePharma Co. They were transfected into SCC9 cells by Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h transfection.

Real-time-polymerase chain reaction (qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was applied for extracting total RNA from OSCC tissues and cells. Complementary DNA (cDNA) was synthesized using the PrimeScript RT reagent kit (Takara). Then, RNA was reverse transcribed using reverse transcription kit (Takara). RT-PCR was performed using the SYBR-Green RT-PCR kit (Bio-Rad Laboratories, Inc.). The expression levels were analyzed using the 2-ΔΔCq method (16). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6 were used as the internal reference for MALAT1 and miR-101, respectively. The primers are shown in Table I.

Western blot analysis. Total proteins were extracted using RIPA lysis buffer (25 mmol/l Tris-HCl + 150 mmol/l NaCl + 1% NP-40 + 1% sodium deoxycholate + 0.1% SDS, pH 7.6; Beyotime Institute of Biotechnology) supplemented with 1% protease inhibitors (100X; Roche Diagnostics) and phenylmethylsulfonyl fluoride (PMSF, 100 mmol/l; Sigma-Aldrich; Merck KGaA). The protein concentration was detected by BCA kit (Bio-Rad Laboratories, Inc.) at 450 nm.

Transwell invasion assay. SCC9 cells were inoculated in a prepared Transwell chamber (8-µm pore size; Corning Inc.) with Matrigel (BD Biosciences), and the cell density of each group was adjusted with serum-free RPMI-1640 culture medium. Briefly, 5x104 SCC9 cells were added to the upper chamber and RPMI-1640 culture medium containing 20% FBS was added to the lower chamber. The cells on the upper chamber were removed with a cotton swab. The cells that migrated to the lower chamber were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature. The number of transmembrane cells from 5 random different fields was selected under a microscope (magnification, x100; SZ61; Olympus).

Dual luciferase activity assay. Through Starbase online software (http://starbase.sysu.edu.cn/index.php), we searched for the potential target miRNAs of MALAT1. The MIMAT number of miR-101-3p is MIMAT0000099 (miRBase). Next, through the TargetScan online software (http://www.targetscan.org/vert_71/), we searched for the potential target genes of miR-101. MALAT1 and EZH2 sequences containing the wild-type (WT) or mutated (MuT)-type miR-101 binding sites were cloned into the pGL3 luciferase reporter vector (pGL3-empty; Promega Corp., respectively). SCC9 cells were co-transfected with miR-101 mimic along with MALAT1-WT/MuT reporter or EZH2-WT/MuT reporter using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), respectively. After a 48-h transfection, luciferase activity was examined using the Dual Luciferase Reporter Assay system (Promega Corp.). The values were normalized to those obtained for pre-miR negative control transfection.
amounts (20 µg) of extracted proteins were separated by 10% SDS-PAGE and then transferred to NC membranes. Then, the NC membranes were blocked with 5% non-fat milk for 1 h at room temperature, and subsequently cultured with the primary antibodies, rabbit monoclonal anti-EZH2 (dilution 1:1,000, ab191080, Abcam), rabbit monoclonal anti-caspase-3 (dilution 1:1,000, ab197202, Abcam) and rabbit monoclonal anti-caspase-8 (dilution 1:1,000, ab25901, Abcam). The membrane was washed with TBST, and incubated with horseradish peroxidase conjugated goat anti-rabbit secondary antibody (ab6721, Abcam) at room temperature for 1 h. Finally, the enhanced chemiluminescence (ECL) was applied for detecting the immune complexes. GAPDH was used as the internal control. The bands were subjected to quantification using the ImageJ imaging processing program (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are expressed as mean ± standard deviation (SD). Each experiment was repeated at least three times. The Student's t-test, one-way analysis of variance (ANOVA) and Pearson product moment correlation coefficient were used for statistical analysis in GraphPad Prism 6.0 software (GraphPad Software, Inc.). Kaplan-Meier survival analysis displayed that the overall survival was shorter in OSCC patients with high expression of MALAT1 compared to that with low expression of MALAT1 (Fig. 1C). We subsequently chose SCC9 for further research because it exhibited the highest MALAT1 expression in the 6 cell lines.

Results

MALAT1 is upregulated in OSCC tissues and cell lines. qPCR was carried out to detect MALAT1 expression in OSCC tissues and cell lines. As shown in Fig. 1A, the expression of MALAT1 was significantly increased in the OSCC tissues compared to that noted in the normal tissues. Then, MALAT1 expression was detected in OSCC cell lines using qPCR. As shown in Fig. 1B, MALAT1 expression was significantly increased in all OSCC cell lines (HSC3, SCC9, SCC15 and SCC25) in comparison with that in normal NHOK cells. OSCC patients were divided into two subgroups (low/high MALAT1 level) using the median level of MALAT1 as a cut-off value. Kaplan-Meier survival analysis displayed that the overall survival was shorter in OSCC patients with high expression of MALAT1 compared to that with low expression of MALAT1 (Fig. 1C). We subsequently chose SCC9 for further research because it exhibited the highest MALAT1 expression in the 6 cell lines.

MALAT1 directly interacts with miR-101 in OSCC cells. The bioinformatics analysis Starbase online software (http://starbase.sysu.edu.cn/index.php) was first performed to detect the potential miRNAs regulated by MALAT1. It was identified that miR-101 was the most probable target miRNA of MALAT1; miR-101 shared complementary binding sites with the 3'-UTR of MALAT1 (Fig. 3A). Dual-luciferase assay was

| Gene     | Primer sequences |
|----------|------------------|
| GAPDH    | Forward primer: 5'-GCACCGTGCAAGGCTGACAAC-3'  |
|          | Reverse primer: 5'-ATGGTGGAAGCAGGCAGT-3'     |
| U6       | Forward primer: 5'-CTGCTTCAGGCGCACA-3'       |
|          | Reverse primer: 5'-AACCGTGCAAGGTGTGGT-3'      |
| lncRNA MALAT1 | 5'-GGGTGTTTACGTAGACCAGAACC-3'   |
|          | Reverse primer: 5'-CTTCCAAAGCCTTCTGCTTC-3'    |
| miR-101-3p | Forward primer: 5'-GGCGGCAATGTTAGACT-3'    |
|          | Reverse primer: 5'-GGAGGTGCTGGATGAGTGA-3'    |
| EZH2     | Forward primer: 5'-TTCAATGCAACACCCCAACT-3'  |
|          | Reverse primer: 5'-GAGAGCGAGGAACTCCT-3'     |

MALAT1, metastasis associated lung adenocarcinoma transcript 1; EZH2, Enhancer of zeste 2 polycomb repressive complex 2 subunit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; lncRNA, long non-coding RNA.
then performed to confirm the bioinformatical prediction. As shown in Fig. 3B, miR-101 mimic significantly downregulated the luciferase activity of MALAT1 3′-UTR-WT in SCC9 cells. However, there was no significantly change in the luciferase activity of MALAT1 3′-UTR-MuT. Furthermore, the result of the qPCR analysis revealed that miR-101 was downregulated in the OSCC tissues when compared with the normal tissues (Fig. 3C). Notably, overexpression of MALAT1 inhibited miR-101 expression, while silencing of MALAT1 expression enhanced miR-101 expression in the SCC9 cells (Fig. 3D). More strikingly, a negative correlation was noted between MALAT1 expression and miR-101 in the OSCC tissues (Fig. 3E). Taken together, these results suggest that MALAT1 directly targets miR-101 in regulating OSCC progression.

Transwell assay is showed in Fig. 4D and E, overexpression of miR-101 inhibited the promotive role of the MALAT1-vector in SCC9 cell invasion, otherwise, miR-101 mimic was able to reverse the suppression of MALAT1-siRNA in regards to cell invasion. Collectively, these findings indicate that MALAT1 facilitates cell proliferation and invasion via suppressing miR-101 in OSCC cells.

EZH2 is a target gene of miR-101 in OSCC cells. TargetScan online software (http://www.targetscan.org/vert_71/) was used to detect the potential target of miR-101. It was found that EZH2 contains a putative binding site for miR-101 as shown in Fig. 5A. The results of dual-luciferase reporter assay indicated that the miR-101 mimic significantly reduced the luciferase activity of EZH2 3′-UTR-WT in the SCC9 cells. However, no significant change was noted in the EZH2 3′-UTR-MuT in SCC9 cells (Fig. 5B). Moreover, the result of qPCR indicated that EZH2 expression was significantly higher in OSCC tissues in comparison with that in the normal tissues (Fig. 5C). Overexpression of miR-101 suppressed EZH2 expression, while silencing of miR-101 expression enhanced EZH2 expression both at the mRNA and protein levels (Fig. 5D and E). These findings suggest that EZH2 is a direct target of miR-101.

EZH2 acts as a downstream gene of MALAT1 in OSCC cells. Then, we investigated whether EZH2 is regulated by MALAT1 through miR-101. As shown in Fig. 6A, EZH2 mRNA and protein expression was significantly enhanced by MALAT1 overexpression and then inhibited by miR-101 mimic. However, silencing of MALAT1 expression inhibited...
EZH2 expression while miR-101 mimic further suppressed EZH2 expression (Fig. 6B). In addition, the results of Pearson's correlation coefficient demonstrated that EZH2 expression was positively associated with MALAT1 expression (Fig. 6C), but negatively correlated with miR-101 expression in OSCC tissues (Fig. 6D). As shown in Fig. 6E, EZH2 overexpression facilitated SCC9 cell viability. The results of the western blot analysis displayed that the levels of caspase-3 and caspase-8 were decreased by the EZH2 plasmid (Fig. 6F). This may provide an added advantage to establish the oncogenic and tumor-suppressive roles of MALAT1 and miR-101 via EZH2.

Discussion

Long non-coding RNAs (lncRNAs) are a class of transcripts with more than 200 nucleotides that do not exhibit protein-coding abilities (17). Recent research has shown that lncRNAs play a pivotal role in the development of many...
Figure 3. MALAT1 directly targets miR-101. (A) Starbase online software was used to predict the putative binding sites of miR-101 with MALAT1. (B) Luciferase activity of MALAT1 3’-UTR-WT (wild-type) or MuT (mutant) in SCC9 cells. **P<0.01, compared with the NC-mimic group. (C) miR-101 expression in OSCC and normal tissues was detected by qPCR. **P<0.01, compared to the normal tissues. (D) qPCR analysis was used to detect the miR-101 expression in SCC9 cells transfected with MALAT1-vector (overexpression) and MALAT1-siRNA (knockdown). **P<0.01, compared with the NC-siRNA or NC-vector group. (E) Correlation between MALAT1 and miR-101 in OSCC tissues was detected by Pearson's correlation coefficient. OSCC, oral squamous cell carcinoma; MALAT1, metastasis associated lung adenocarcinoma transcript 1.

Figure 4. MALAT1 facilitates OSCC cell viability and invasion via modulating miR-101. (A and B) The viability of SCC9 cells following transfection was detected by CCK-8 assay. *P<0.05, compared with the +miR-101-mimic transfected group. (C) Western blot analysis was used to detect the caspase-3 and caspase-8 levels in SCC9 cells in the different cell groups. (D and E) Transwell assay analysis was used to detect SCC9 cell invasion in the different cell groups. **P<0.01, compared with the +miR-101-mimic transfected group. OSCC, oral squamous cell carcinoma; MALAT1, metastasis associated lung adenocarcinoma transcript 1.
tumors, but the related mechanism remains to be investigated (18). In this study, we revealed that metastasis associated lung adenocarcinoma transcript 1 (MALAT1) was upregulated in oral squamous cell carcinoma (OSCC) tissues and cell lines (HSC3, SCC9, SCC15 and SCC25).

MALAT1 is an important lncRNA in tumor progression. Previous studies have shown that MALAT1 is upregulated in colorectal cancer (CRC), pancreatic ductal adenocarcinoma and head and neck squamous cell carcinoma (19-21). Furthermore, Xu et al. displayed that MALAT1 overexpression promoted the viability and migration of CRC (22). Pan et al. found that overexpression of MALAT1 enhanced the invasion and migration of hepatocellular carcinoma (23). Huang et al. implicated the role of MALAT1 in facilitating the angiogenesis of breast cancer (24). Zhu et al. found that MALAT1 displayed facilitating effect on gastric cancer cell proliferation and metastasis (25). Moreover, Lin et al. displayed that MALAT1 was upregulated in gallbladder cancer and its high expression was associated with poor prognosis (26). Stone et al. showed high expression of MALAT1 in breast cancer and MALAT1 was associated with tumor development (27). Moreover, Zhou et al. revealed that MALAT1 was elevated in OSCC and promoted tumor growth and metastasis (28). Our findings revealed that MALAT1 enhanced OSCC cell proliferation and invasion, which supporting previous observations that MALAT1 acts as an oncogene in OSCC development (29). The results of this study also displayed that the levels of caspase-3 and caspase-8 were decreased by MALAT1 overexpression, while increased by silencing of MALAT1. In the present study, we chose to investigate caspase-3 and caspase-8 but not caspase-9, which may be the limitation of the present study.

As the development of RNA-sequencing technologies, many non-coding RNAs have been identified. lncRNAs possess important functions in development, immunology and cancer. For example, lncRNA CASC2 was found to be downregulated in OSCC and markedly inhibited cell proliferation via targeting microRNA-21 (30). On the other hand, Zhang et al. showed that lncRNA PAPAS conferred a promotive effect on OSCC development as an oncogene by modulating the TGF-β1 signaling pathway (31). In addition, lncRNA LEF1-AS1 was found to act as an oncogene in OSCC progression and silencing of LEF1-AS1 suppressed cell proliferation, migration and tumor growth through the Hippo signaling pathway (32).

Recently, a growing number of reports have suggested that lncRNAs act as ‘sponges’ to bind specific miRNAs and regulate their function. Previous research has established that MALAT1 promoted hepatocellular carcinoma cell migration and invasion by targeting miR-142-3p (33). Chang and Hu identified the biological function of MALAT1 in promoting OSCC development via targeting the miR-125b/STAT3 axis (29). Here, we showed that MALAT1 is a target of miR-101 by bioinformatics analysis and luciferase reporter assays. Thus, our research is the first to report that MALAT1 regulates OSCC proliferation and invasion by targeting miR-101.

By using TargetScan, we predicted Enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) as a direct target of miR-101. As the development of RNA-sequencing technologies, many non-coding RNAs have been identified. lncRNAs possess important functions in development, immunology and cancer. For example, IncRNA CASC2 was found to be downregulated in OSCC and markedly inhibited cell proliferation via targeting microRNA-21 (30). On the other hand, Zhang et al. showed that lncRNA PAPAS conferred a promotive effect on OSCC development as an oncogene by modulating the TGF-β1 signaling pathway (31). In addition, IncRNA LEF1-AS1 was found to act as an oncogene in OSCC progression and silencing of LEF1-AS1 suppressed cell proliferation, migration and tumor growth through the Hippo signaling pathway (32).

Figure 5. EZH2 is a target of miR-101. (A) Prediction the putative binding sites of miR-101 with EZH2. (B) Luciferase activity of EZH2 3'-UTR-WT (wild-type) or MuT (mutant) in SCC9 cells. **P<0.01, compared with the NC mimic or NC inhibitor group. (C) Measurement of EZH2 expression in OSCC and normal tissues by qPCR. *P<0.05, compared with the NC mimic or NC inhibitor group. OSCC, oral squamous cell carcinoma; EZH2, Enhancer of zeste 2 polycomb repressive complex 2 subunit.
regulate EZH2 expression by modulating miR-101 expression. Overexpression of MALAT1 significantly promoted EZH2 expression, while treatment with the miR-101 mimic attenuated EZH2 expression. Conversely, knockdown of MALAT1 inhibited EZH2 expression, while transfection with the miR-101 mimic further suppressed EZH2 expression.
In conclusion, the present study aimed to discover the biological function and molecular mechanism of IncRNA MALAT1 in OSCC development. The findings showed that MALAT1 was upregulated, while miR-101 was downregulated in OSCC tissues and cell lines. MALAT1 modulated OSCC progression by negatively regulating miR-101. Moreover, EZH2 was determined as the target of miR-101 by bioinformatics analysis. Taken together, MALAT1 regulates EZH2 expression by modulating miR-101, which may be considered as a novel target in OSCC treatment.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions
LX first author contributed significantly to analysis and manuscript preparation. WW as the co-first author performed the data analyses and wrote the manuscript. HX as the corresponding author contributed to the conception of the study. JZ, SL and XY helped perform the analyses with constructive discussions. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate
The study was approved by the Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University. Signed written informed consents were obtained from the patients and/or guardians.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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