Original Article
Long non-coding RNA TUG1 promotes progression of oral squamous cell carcinoma through upregulating FMNL2 by sponging miR-219

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Abstract: Oral squamous cell carcinoma (OSCC) is a prevalent oral disease with a high morbidity and mortality rate. Several long non-coding RNAs (lncRNAs) were identified as important regulators of carcinogenesis. However, the pathogenic implications of TUG1 in OSCC are still unclear. In the present study, the expression of TUG1 was increased in OSCC cells. Knockdown of TUG1 inhibited cell proliferation, migration, and invasion, and induced cell cycle arrest at G0/G1 phase, whereas overexpression of TUG1 exerted the opposite effect on OSCC cells. A reciprocal repressive interaction between TUG1 and miR-219 was found, and miR-219 inhibition abolished the tumor-suppressive effect of TUG1 knockdown on cell growth and motility. Furthermore, bioinformatics analysis and luciferase reporter assay showed that FMNL2 was a direct target of miR-219. Restoration of FMNL2 abrogated the miR-219-induced inhibition of cell proliferation, cell cycle progression, migration, and invasion. Besides, overexpression of TUG1 promoted tumor growth and metastasis in vivo. Clinically, the expression of TUG1 and FMNL2 were increased, but miR-219 was decreased in primary tumors compared to non-tumor tissues. Both the upregulated TUG1, and FMNL2 and the downregulated miR-219 was associated with advanced stage of OSCC and poor overall survival. Notably, multivariate analyses confirmed that FMNL2 was an independent risk factor for OSCC. In conclusion, our data revealed that TUG1 confers oncogenic function in OSCC and TUG1/miR-219/FMNL2 axis may be a novel therapeutic strategy in this disease.

Keywords: TUG1, oral squamous cell carcinoma, miR-219, FMNL2, metastasis, prognosis

Introduction
Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide with increasing incidence in the recent years [1, 2]. Despite the advances in treatment of OSCC, it is still unsatisfactory that the 5-year survival rate remains at less than 50% [3]. Previous studies have demonstrated multiple genetic and epigenetic abnormality in OSCC, but the underlying mechanism responsible for OSCC tumorigenesis are not well understood.

It has been recognized that the vast majority of human transcripts are non-coding RNAs (ncRNAs), including microRNA (miRNAs, <200 nucleotides in length) and long ncRNAs (lncRNAs, >200 nucleotides in length) [4]. Increasing evidences indicated that the dysregulated IncRNAs and miRNAs were correlated with tumor progression and cancer-related cellular process, including proliferation, apoptosis, migration, and invasion, etc [5-8]. It is recently reported that IncRNAs functioned as molecular sponges to competitively suppress miRNAs, which could regulated oncogenes or tumor suppressors by binding to 3'-untranslated region (3'-UTR) of mRNAs [9, 10]. For example, MALAT1 mediated the expression of MCL-1 as a competing endogenous RNA (ceRNA) for miR-363-3p in gallbladder carcinoma cells [11]. Overexpression of GAS5 inhibited the expression of miR-222 and consequently increased the expression of tumor suppressor Bcl-2-modifying factor and PLXNC1 [12]. Among these IncRNAs, TUG1 was found to be commonly upregulated in various...
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Table 1. Correlations between the clinicopathological features and the expression of TUG1, miR-219, and FMNL2 in OSCC

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*Union for International Cancer Control; never, no smoking history, quit, stop smoking no more than ten years; now, smoker; L, low expression; H, high expression.

types of tumors, including colorectal cancer (CRC), bladder cancer, hepatocellular carcinoma (HCC), and cervical cancer [13-16]. It has been demonstrated that TUG1 regulated tumor cell growth and motility through mediating different miRNAs [17-19]. However, the clinical significance and biological function of TUG1 in OSCC are not fully elucidated.

FMNL2, a member of the diaphanous-related formins, act as effectors of Rho family guanosine triphosphatases (GTPases) and play critical roles in carcinogenesis [20, 21]. The expression of FMNL2 was frequently increased in several cancers and positively associated with metastasis [21-23]. Moreover, the expression of FMNL2 could be regulated by many miRNAs, such as miR-137, miR-34a, and miR-206 [24-26]. It has attracted our attention that the expression and function of FMNL2 in OSCC.

In the present study, we found that TUG1 exerted its oncogenic function via regulating miR-219 and the downstream target gene FMNL2. The dysregulated TUG1, miR-219, and FMNL2 were significantly associated with lymph node metastasis (LNM), advanced phenotype of OSCC, and patients’ survival. Therefore, the dysfunction of TUG1/miR-219/FMNL2 may be a novel mechanism and a promising therapeutic strategy for oral malignancy.
Material and methods

Cell lines and tumor samples

The human OSCC cell lines, HN4, HN6, SCC-25, CAL-27, and a normal human oral keratinocyte cell line (NHOK) were obtained from Cell Bank of the Chinese Academy of Science (Shanghai, China) and maintained in DMEM (HyClone, South Logan, UT, USA) supplemented with 10% FBS (HyClone), and 100 μg/ml penicillin/streptomycin (Bio Light, Shanghai, China). All the cells were incubated at 37°C in a humidified atmosphere with 5% CO2.

Tissue specimens (including tumors and matched adjacent non-tumor tissues) were obtained from forty-six OSCC patients who were diagnosed and treated at the Department of Oral and Maxillofacial Surgery, China Medical University School of Stomatology between 2010 and 2012. The protocol of this study was approved by the Ethics Committee of the China Medical University and informed consents were provided by all patients. Both tumors and non-tumor tissues were recognized by pathologists and the TNM stage were defined according to the Union for International Cancer Control (UICC). Follow-up data were collected by regular visit or telephone interview. All clinicopathological characteristics of patients were summarized in Table 1.

Quantitative real-time PCR (qPCR)

The total RNA was isolated from tissues by using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). High Capacity cDNA RTKit (ABI, Foster City, CA, USA) was used for TUG1 and FMNL2 reverse transcription reaction and the Taqman MicroRNA Reverse Transcription Kit (ABI) was used for miR-219 analysis. QPCR was performed using TaqMan Universal Master Mix II (ABI) with a 7300 Real-Time PCR System (ABI). The relative expression of each gene was normalized using the 2-ΔΔCt method relative to the expression of U6 or GAPDH. The following primers were used: TUG1 (5'-TTCTACAACTTAC-TACTGAC-3', 5'-GGAGGTAAGGGCACCATC-3'), FMNL2 (5'-GCTATGAACTTACCTCTGACA-3', 5'-AACAGGCGCTCGAATTTCTT-3'), miR-219 (5'-AACCTCAAGCGGTGGTTATGTCACCTAAGGCAAT-3', 5'-CTCAACTGTTGTCGTGGGA-3').

Lentiviral vector construction and transfection

The TUG1 sequence was synthesized and subcloned into the pcDNA3.1 vector (Invitrogen) to generate pc-TUG1 vector and the empty pcDNA3.1 vector was used as a control (pc-DNA). A short hairpin RNA (shRNA) sequences were designed by Hanbio (Shanghai, China) to target human TUG1 and named as sh-TUG1 (negative control was named sh-ctrl) and plasmids were co-transfected into cells along with packaging plasmids. Following infection for 48 h, cells were selected with puromycin (Sigma, St.Louis, MO, USA). To assess the effect of TUG1 on tumor growth and metastasis in vivo, the TUG1 gene was cloned in to a lentivirus vector LV-GFP-Puro. The stable transfection cells were established by puromycin selection and the stably expressing TUG1 cells and control cells were named as LV-TUG1 and LV-NC, respectively. The miR-219 mimic, mimic negative control (M-NC), miR-219 inhibitor, and inhibitor negative control (I-NC) were synthesized by RiboBio (Guangzhou, China) and transfection were performed using Lipofectamine 3000 reagent (Invitrogen). FMNL2 expression vector without 3'-UTR (pc-FMNL2) and empty vector (pc-DNA) were obtained from Genechem (Shanghai, China) and transferred into cells using lipofectamine 3000 (Invitrogen) according to manufacturer’s protocols.

Cell proliferation assay

Cell proliferation was determined using the MTT method. After transfection, cells were seeded into 96-well culture plates (3000 cells/well) and incubated at 37°C overnight. At indicated time points (24, 48, 72, or 96 h), 100 μl of MTT (5 mg/ml, Sigma) solution was added to each well and cells were incubated for another 4 h. Then, the MTT was discarded and 150 μl DMSO was added to stop the reaction. The spectrometric absorbance at 490 nm was measured using a microplate reader (BioRad, Hercules, CA, USA). The experiment was run in triplicate for each sample.

Cell cycle analysis

Cells were collected after transfection and washed twice with cold PBS, followed by fixed in ice-cold 70% ethanol at 4°C overnight. Then, cells were resuspended in FACS solution containing RNase (0.1 g/l, Sigma) and propidium iodine (PI, 50 μg/ml) in the dark at 37°C. Then, FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA) and ModFit software (Becton Dickinson) was used to analyze cells and flow cytometry data, respectively.
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**In vitro migration and invasion assay**

Cell migration and invasion were evaluated using Transwell migration chambers (8 μm pore size, Corning, New York, USA). For the invasion assays, cells were plated in the upper chamber of each insert, which had previously been coated with 150 μg of Matrigel (BD Biosciences, San Jose, CA, USA). 4×10⁴ cells were trypsinized, resuspended in FBS-free medium, and seeded in the upper chamber. Medium containing 600 μl 10% FBS was added to the lower chamber in the migration assay, while 20% FBS medium in the invasion assay. After incubation at 37°C for 24 h, cells on the upper membranes of the insert were removed, and the cells that migrated or invaded to the lower membranes of the inserts were fixed, stained, and counted. The experiment was performed in triplicate.

**Plasmid construction and luciferase reporter assay**

We used the bioinformatics databases to search for potential miRNAs that can bind to TUG1. The TUG1 fragment containing the miR-219 binding site was amplified and cloned into the pmirGLO Vector (Promega, Madison, Wisconsin, USA) to generate the wild type pmirGLO-TUG1 reporter vector (TUG1-WT). The putative binding site of miR-219 in TUG1 was mutated by using a QuikChange Site Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA) to synthesize mutant type pmirGLO-TUG1 vector (TUG1-Mut). Cells were co-transfected with the pmirGLO vector with either TUG-WT/TUG1-Mut and miR-219 mimic/M-NC. Luciferase reporter assay was performed using the Dual-Luciferase Reporter Assay System (Promega).

To confirm the direct regulating relationship between miR-219 and FMNL2, the wild type 3’-UTR of the FMNL2 mRNA (FMNL2-WT) was amplified and cloned into the pGL3-basic vector (Promega). The Site-Directed Mutagenesis System (Invitrogen) was used to construct a mutant 3’-UTR (FMNL2-Mut). When grew to 60-70% confluence, cells were transfected with miR-219 mimic or M-NC, along with FMNL2-WT or FMNL2-Mut. After incubation for 48 h, the cells were harvested and assayed using the Dual-luciferase reporter assay system (Promega) according to the manufacturer’s instructions.

**Western blot analyses**

Cells were lysed with RIPA buffer (Applygen, Beijing, China) and protein lysates were separated by 10% SDS-PAGE, followed by transferring to polyvinylidene difluoride (PVDF) membranes (Millipore, Boston, MA, USA). Then, the membranes were blocked with 5% free-fat milk in TBS containing 0.1% Tween (TBST) at room temperature for 2 h. Membranes were incubated overnight with anti-FMNL2 antibody (Abcam, Cambridge, UK), anti-β-actin antibody (Santa Cruz Bio., Santa Cruz, CA, USA) at 4°C overnight. After washing with TBST twice, the membranes were incubated with secondary antibodies at 37°C for 1 h and protein bands were visualized with the ECL detection system (GE, Fairfield, CT, USA).

**Animal experiments**

All animal experiments were approved by the Animal Research Committee at China Medical University and were carried out in accordance with established International Guiding Principles for Animal Research. To form xenograft tumors, CAL-27 cells (5×10⁶) transfected with LV-NC or LV-TUG1 were subcutaneously injected into the right flank of 4-weeks old female athymic nude mice. Five mice were used for each treatment group. Tumor volume was monitored every four days and calculated according to the following formula: volume = (length × width²)/2. Tumor xenografts were harvested at 35 days after cell transplantation.

For evaluating metastasis in vivo, twenty female NOD/SCID mice (4-6 weeks) were used. 2×10⁶ CAL-27 cells treated with LV-NC or LV-TUG1 were injected in PBS intravenously in the mice tail vein (ten mice for each group). 8 weeks later, the mice were sacrificed and livers and lungs were obtained for checking tumor nodes. Then, lung tissues were fixed for paraffin-embedded section (4 μm), and stained with hematoxylin and eosin (HE).

**Statistical analysis**

Both SPSS 16.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism 5.0 (Graphpad, La Jolla, CA, USA) were used for statistical analysis. All the data obtained from at least three times of experiments were presented as the mean ± SEM. Student t-test was used to
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Figure 1. TUG1 contributes to cell proliferation, cell cycle progression, migration, and invasion. (A) QPCR assay was used to measure the expression of TUG1 in OSCC cells and the normal control NHOK cells. (B and C). QPCR analysis was used to determine the transfection efficiency of sh-TUG1 (B) and pc-TUG1 (C). (D) MTT assay showed that sh-TUG1 significantly decreased proliferation rate of CAL-27 cells and pc-TUG1 increased proliferation of HN4 cells. (E) By flow cytometry analysis, sh-TUG1 induced an increasing of cells in G0/G1 phase and a reduction of cells in S phase; pc-TUG1 increased the percentage of cells in S phase. (F) Knockdown of TUG1 inhibited cell migration in CAL-27 cells and ectopic expression of TUG1 enhanced cell migration in HN4 cells. (G) Cell invasive ability was inhibited by sh-TUG1, while increased by pc-TUG1. *P<0.05; **P<0.01.

estimate the significant differences between groups. Kaplan-Meier method and log-rank test were used in overall survival (OS) analysis. To evaluate independent prognostic factors correlated with survival, a univariate and multivariate Cox proportional hazards regression analysis was performed. Relationships between two moleculars were explored by Pearson correlation analysis. P<0.05 was considered as statistically significant.
Results

**TUG1 contributed to OSCC proliferation and invasion**

Firstly, we determined the endogenous levels of TUG1 in OSCC cells. We found that the expression of TUG1 in OSCC cells were significantly higher than that in control NHOK cells (P<0.05, Figure 1A). We knockdown TUG1 in CAL-27 cells, which expressing relatively high level of TUG1, and ectopic expression of TUG1 in HN4 cells, which expressing relatively low level of TUG1. The expression of TUG1 was significantly downregulated and upregulated in sh-TUG1 and pc-TUG1 transfected cells, respectively (Figure 1B and 1C).

The results of MTT assay showed that knockdown of TUG1 in CAL-27 cells significantly inhibited cell proliferation when compared with sh-ctrl group and blank control group. In contrast, cell viability was significantly increased in pc-TUG1-treated cells in comparison with the pc-TUG1.
DNA and blank control-treated cells (Figure 1D). In flow cytometry assay, TUG1 inhibition increased the cells at the G0/G1 phase and decreased the cells at the S phase, while TUG1 overexpression promoted the cell cycle progression from G0/G1 phase to S phase (Figure 1E). We next performed cell migration and invasion transwell assays to explore the effect of TUG1 on cell motility. The results showed that downregulation of TUG1 decreased the migratory and invasive ability of CAL-27 cells compared with corresponding control cells (Figure 1F and 1G). On the contrary, upregulation of TUG1 promoted cell migration and invasion in HN4 cells (Figure 1F and 1G). These results suggest that TUG1 plays an oncogenic role in OSCC tumorigenesis by enhancing cell growth and motility.

**MiR-219 abolished the effect of TUG1 on cell growth and invasion**

Previous studies have shown that IncRNAs function as ceRNAs or molecular sponges to regulate miRNAs. Using the starBase v2.0 and RegRNA 2.0, miR-219 was found to potentially bind to TUG1 (Figure 2A). The results showed that the expression of miR-219 was increased...
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Figure 2. Ectopic TUG1 expression promotes tumor growth and metastasis in vivo. A and B. Representative images of tumors formed in nude mice (n = 5 for each group); tumor volumes were significantly increased in LV-TUG1 treated mice. C. The tumor weight was markedly higher in mice transfected with LV-TUG1 than that in LV-NC group. D-F. The expression of TUG1, miR-219, and FMNL2 mRNA levels were measured in mice by qPCR method. G. The protein levels of FMNL2 were elevated after ectopic TUG1 in mice. H. Lung metastasis model generated by injecting tumor cells into the tail veins of mice; HE staining showing the metastasis foci in lungs in each group; *P<0.05; **P<0.01.

in sh-TUG1 transfected cells compared to control cells (Figure 2B). TUG1 expression was repressed after ectopic expression of miR-219, whereas elevated after inhibition of miR-219 (Figure 2C). To verify the direct binding relationship between TUG1 and miR-219, a luciferase activity assay was performed. Co-transfection of miR-219 and TUG1-WT significantly reduced the luciferase activity, whereas co-transfection of M-NC and TUG1-WT did not change the luciferase activity (Figure 2D).

Furthermore, to examine the effect of miR-219 combined with TUG1 silencing on OSCC cell behavior, CAL-27 cells were divided into two groups: transfected with I-NC and sh-TUG1, and transfected with inhibitor and sh-TUG1. As shown in Figure 2E, miR-219 inhibition abrogated the sh-TUG1 induced suppressing of cell proliferation. The percentage of cells were decreased at the G0/G1 phase and increased at the S phase after co-transfected CAL-27 cells with miR-219 inhibitor and sh-TUG1 (Figure 2F). Besides, miR-219 inhibitor impaired the reduction of cell migration and invasion induced by sh-TUG1 (Figure 2G and 2H). In HN4 cells, overexpression of miR-219 abolished the promotive effect of TUG1 on cell proliferation and cell cycle transition from G0/G1 to S phase (Figure 2I and 2J). Ectopic expression of miR-219 suppressed the TUG1 induced increasing of cell migratory and invasive ability (Figure 2K and 2L). These findings suggest that TUG1/miR-219 axis may be involved in OSCC progression by regulating cell proliferation, cell cycle distribution, migration, and invasion.

FMNL2 is a direct target of miR-219

It is well known that miRNAs through targeting its target genes associated with carcinogenesis to regulate tumor-related biological process. Herein, by using bioinformatic tools (microRNA, TargetScan, miRDB, and PicTar), we found that FMNL2 may be a target gene of miR-219 (Figure 3A). The luciferase assay showed the miR-219 mimic decreased the luciferase activity of FMNL2-WT in both CAL-27 and HN4 cells, but did not affect the luciferase activity of FMNL2-Mut (Figure 3B and 3C). Moreover, the mRNA and protein expression of FMNL2 were obviously suppressed by miR-219 mimic in CAL-27 cells, and this inhibition could be abolished by co-transfected with a pc-FMNL2 vector (lacking
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Figure 3. It should be clear that whether TUG1 can regulate the expression of FMNL2. We found that TUG1 silencing significantly decreased the mRNA and protein levels of FMNL2, as well as abolished the miR-219 inhibitor induced the expression of FMNL2 mRNA and protein (Figure 3F and 3G). Taken together, these results suggest that FMNL2 is a downstream target of miR-219.

Overexpression of FMNL2 impaired the tumor suppressive effect of miR-219

To verify whether the regulatory effects of miR-219 on cell proliferation, migration and invasion are mediated by FMNL2, miR-219-overexpressing CAL-27 cells were co-transfected with pc-FMNL2 (without 3'-UTR) or control pc-DNA. As shown in Figure 3H and 3I, restoration of FMNL2 could abrogate miR194-induced reduction of cell proliferation and cell cycle progression from G1 to S phase. Likewise, both cell migration and invasion were inhibited by miR-219 mimic, while enhanced by ectopic expression of FMNL2 (Figure 3J and 3K). Overexpression of FMNL2 impaired the inhibitory effect of miR-219 mimic on cell migration and invasion, respectively (Figure 3J and 3K). Thus, these results indicate that FMNL2 is a functional target of miR-219 and plays oncogenic role by increasing cell proliferation and invasion.

TUG1 promotes tumor growth and metastasis in vivo

To confirm the effect of TUG1 overexpression in vivo, a mouse xenograft model was established. CAL-27 cells were transfected with LV-NC or LV-TUG1 and subcutaneously injected into the nude mice (n=5 for each group). As shown in Figure 4A and 4B, the results showed that the TUG1 overexpression group displayed...
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A markedly higher growth rate and tumor volume when compared with control group. The tumor weight from the LV-TUG1 transfected group was significantly elevated compared with the LV-NC treated group (Figure 4C). We determined the expression of TUG1, miR-219 and FMNL2 in xenograft tumors. The result of qPCR confirmed that the expression of TUG1 was significantly upregulated in LV-TUG1 transfected mice (Figure 4D). Besides, miR-219 was decreased and FMNL2 was increased in TUG1 overexpression group (Figure 4E and 4F). The protein levels of FMNL2 were increased in LV-TUG1 transfection group (Figure 4G).

In order to explore the effect of TUG1 in regulating metastasis in vivo, we injected CAL-27 cells stably transfected with LV-NC or LV-TUG1 into the tail vein of NOD/SCID mice to construct experimental metastasis models. Eight weeks later, the mice were executed and lung and liver were harvested for examining the tumor metastasis. As a result, no visible metastasis nodules was found in the liver, as well as in the lungs of LV-NC treated mice. However, we found that three mice developed tumor nodes in the lungs of LV-TUG1 treated group. HE staining confirmed that large lung metastasis foci was observed in the LV-TUG1 transfected mice when compared with the LV-NC induced micrometastasis foci (Figure 4H).

The expression status of TUG1, miR-219, and FMNL2 in OSCC samples

Considering the dysregulated TUG1, miR-219, and FMNL2 were correlated with OSCC cell growth and migration in vitro and in vivo, we measured the expression of these three molecules in clinical samples. The expression of TUG1 was significantly upregulated in primary tumors compared to non-tumor tissues (1.566±0.828 vs. 1.152±0.603; P=0.0002; Figure 5A). miR-219 was markedly decreased in OSCC tissues compared to noncancerous tissues (0.541±0.353 vs. 0.788±0.579; P=0.0017; Figure 5B). The mRNA expression of FMNL2 in tumors were significantly higher than that in non-tumor tissues (2.425±0.961 vs. 1.511±1.004; P<0.001; Figure 5C).

Furthermore, we found that the expression of TUG1 was negatively correlated with miR-219 but not attain statistical significance (Pearson r=-0.264, P=0.07; Figure 5D). A significantly inverse correlation was found between miR-219 and FMNL2 (Pearson r=-0.548, P<0.001; Figure 5E). The mRNA levels of FMNL2 were positively associated with TUG1 in tumors (Pearson r=0.298, P=0.039; Figure 5F).

Dysregulated TUG1/miR-219/FMNL2 were associated with tumor progression and prognosis

Correlations between clinicopathological characteristics and the expression of TUG1, miR-219, and FMNL2 in OSCC patients were analyzed, and the mean value of the expression levels of each gene in tumors was used as cut-off to classify cases into two groups (high- or low-expression). As shown in Table 1, the upregulated TUG1 was correlated with lymph node metastasis (LN, P=0.043) and advanced TNM stage (P=0.02). The expression of miR-219 was significantly lower in cases with LNM (P=0.027) and advanced tumor stage (P=0.024), respectively. In addition, the mRNA levels of FMNL2 in tumors with LNM and advanced TNM stage were markedly increased (P=0.004, and 0.006; respectively). These results suggested that the dysregulation of TUG1, miR-219, and FMNL2 were associated with the progression of OSCC.

The results of Kaplan-Meier analysis showed that the high expression of TUG1, the low level of miR-219, and the increased FMNL2 were significantly correlated with the poor OS of patients (p=0.018, 0.011, and 0.0004, respectively; Figure 5G-I). The impact of these three molecules on survival were also confirmed be univariate analysis (Table 2). Besides, LNM (P=0.018, hazard ration (HR)=2.692, confidence interval (CI): 1.181-6.133) and TNM stage (P=0.041, HR=2.460, CI: 1.038-5.832) were also found to be positively correlated with poor prognosis by using univariate analysis (Table 2). However, among these significant prognostic factors, multivariate analysis showed that only the expression of FMNL2 (P=0.027, HR=3.256, CI: 1.144-9.268) was the independent prognostic factor for OS.

Discussion

Accumulating studies have showed that dysregulated IncRNAs is closely related with the initiation and progression of cancer and may be used as diagnostic or prognostic marker [27]. Herein, we found that the expression of TUG1
TUG1 promotes oral cancer through miR-219/FMNL2

Table 2. Clinical characteristics of OSCC patients correlate with overall survival by Cox proportional hazard regression analysis

<table>
<thead>
<tr>
<th>Features</th>
<th>HRs</th>
<th>P</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (&lt;55/≥55)</td>
<td>1.850</td>
<td>0.172</td>
<td>0.766-4.468</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>1.688</td>
<td>0.218</td>
<td>0.735-3.878</td>
</tr>
<tr>
<td>T stage (T1-2/T3-4)</td>
<td>1.480</td>
<td>0.413</td>
<td>0.579-3.783</td>
</tr>
<tr>
<td>N stage (NO/N1-2)</td>
<td>2.692</td>
<td>0.018</td>
<td>1.181-6.133</td>
</tr>
<tr>
<td>TNM stage (I, II/III, IV)</td>
<td>2.460</td>
<td>0.041</td>
<td>1.038-5.832</td>
</tr>
<tr>
<td>Smoking (never/quit, now)</td>
<td>1.699</td>
<td>0.244</td>
<td>0.697-4.141</td>
</tr>
<tr>
<td>Diff (well/mod, poor)</td>
<td>1.093</td>
<td>0.828</td>
<td>0.488-2.452</td>
</tr>
<tr>
<td>TUG1 level (L/H)</td>
<td>2.679</td>
<td>0.023</td>
<td>1.149-6.247</td>
</tr>
<tr>
<td>miR-219 level (H/L)</td>
<td>2.984</td>
<td>0.015</td>
<td>1.240-7.178</td>
</tr>
<tr>
<td>FMNL2 level (L/H)</td>
<td>4.959</td>
<td>0.001</td>
<td>1.903-12.920</td>
</tr>
</tbody>
</table>

Multivariate

<table>
<thead>
<tr>
<th>Features</th>
<th>HRs</th>
<th>P</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMNL2 level (L/H)</td>
<td>3.256</td>
<td>0.027</td>
<td>1.144-9.268</td>
</tr>
</tbody>
</table>

HRs, hazard ratios; CI, confidence interval; F, female; M, male; Diff, differentiation; mod, moderate; L, low expression; H, high expression; P<0.05 are present in bold.

was upregulated in OSCC cells, suggesting that TUG1 may have roles in OSCC. The cell proliferation, cell cycle progression, migration, and invasion were significantly inhibited by TUG1 knockdown, while enhanced by TUG1 overexpression. These findings were in consistent with previous studies regarding on glioblastoma, renal cell carcinoma, CRC, and lung cancer [18, 28-30]. A recent study also reported that knockdown of TUG1 inhibited colony formation and invasion of OSCC cells [31]. In addition, we found that overexpression of TUG1 contributed to tumor growth and metastasis in vivo. Our data expanded the knowledge of TUG1 as a oncogenic regulator in the development of OSCC.

Subsequently, we focused on the underlying mechanism of TUG1 induced alterations of cellular process. Given that IncRNAs can function as molecular sponges to competitively inhibit miRNAs, we searched for candidate miRNAs which may be involved in the TUG1 mediated cell growth and invasion. As a result, we found that TUG1 and miR-219 could affect each other’s expression. Further analysis confirmed that luciferase activity of the reporter containing TUG1-WT was decreased in miR-219 mimic transfected cells. Functionally, miR-219 inhibition abrogated the effect of TUG1 knockdown on OSCC cells by promoting cell proliferation, G1/S transition, migration, and invasion, whereas miR-219 upregulation repressed cell growth and motility, suggesting the tumor-suppressive role of miR-219. Several previous studies also suggested miR-219 as a tumor suppressor in other tumors [32-36]. We found that miR-219 could arrest cell cycle at the G1 to S phase, this phenomenon could be observed in HCC analysis [37]. Our data was the first study to show the regulatory effect of TUG1/miR-219 axis on OSCC cells. Several other miRNAs have been shown to bind TUG1 and were involved in TUG1 regulated tumor progression. For example, a reciprocal repression between TUG1 and miR-145 was found in bladder cancer [17]. MiR-299 was upregulated after knockdown of TUG1 in glioblastoma cells [18]. Overexpression of miR-335 impaired the promotive effect of TUG1 on migration of osteosarcoma cells [19]. Thus, it hints that TUG1 may be through targeting different miRNAs to regulate cell growth and invasion in different human malignancies.

In our study, the expression FMNL2 was repressed by miR-219 and overexpression of FMNL2 impaired the inhibitory effect of miR-219 on cell viability and motility, suggesting the oncogenic function of FMNL2 in OSCC. Besides miR-219, FMNL2 was found to be repressed by miR-34a, miR-206, and miR-613, respectively [25, 26, 38]. Moreover, we found that TUG1 could promote the mRNA and protein expression of FMNL2 by sponging miR-219; suggesting the regulatory function of TUG1/miR-219 axis on FMNL2. In vivo analysis also confirmed a reduction of miR-219 expression and an increasing of FMNL2 mRNA and protein levels in TUG1 overexpressing mice. Interestingly, FMNL2 was known as a positive regulator of epithelial mesenchymal transition (EMT) [21], and TUG1 was reported to have promoted EMT in bladder cancer and CRC [17, 29]. Thus, we speculated that EMT might be regulated by TUG1 through miR-219/FMNL2 pathway in carcinogenesis.

Clinically, the upregulated TUG1 indicated the aggressive phenotype of OSCC and poor prog-
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nosis, which was in consistest with previous studies [13-17, 30, 39]. The expression of TUG1 was negatively correlated with miR-219, although the difference failed to attain statistical significance. There may be other mechanisms involved in regulating miR-219, such as DNA methylation [40]. Our data showed that the reduced miR-219 was more frequently observed in advanced stage of tumors, and the lower miR-219 was associated with poor survival. Similar results were found in gastric cancer, HCC, astrocytoma, and prostate cancer [35, 37, 41, 42]. The low expression of FMNL2 was strongly associated with the high miR-219 in tumors, supporting the negative correlation between miR-219 and FMNL2 in primary tumors. The expression of FMNL2 in OSCC tissues have not been reported yet. However, by analyzing datasets from Oncomine, we found that the mRNA levels of FMNL2 were significantly elevated in tongue squamous cell carcinoma (P=1.03×10^{-4}). This phenomenon was also observed in our study by qPCR validation. The higher expression of FMNL2 was strongly associated with LNM and advanced tumor stage, supporting FMNL2 as a positive regulator of metastasis [21-23]. Multivariate analysis indicated an independent prognostic significance of FMNL2 expression. Further investigation is required for verifying TUG1, miR-219, and FMNL2 as prognostic markers in human oral cancer. Taken together, we suggested that the dysregulated TUG1, miR-219, and FMNL2 were the possible reasons for the progression of OSCC.

In this study, we revealed that TUG1 could promote tumor growth and metastasis through acting as a molecular sponge of miR-219, ultimately increasing the expression of FMNL2, which could be used as a prognostic factor in OSCC. Overall, the dysfunction of TUG1/miR-219/FMNL2 axis may be a novel mechanism responsible for OSCC and a promising therapeutic stragety.

Disclosure of conflict of interest

None.

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