Abstract. Long non-coding RNAs (lncRNAs) have emerged as critical regulators of tumor progression. However, the function and mechanism of lncRNA NEAT1 in oral squamous cell carcinoma (OSCC) are unclear. In the present study, NEAT1 was significantly upregulated in OSCC cells and tissues. High expression of NEAT1 was correlated with advanced TNM stage and poor survival of patients. Using bioinformatics prediction and experimental analysis, we determined that NEAT1 could negatively regulate the expression of miR-365. The expression of miR-365 was decreased in OSCC tissues and inversely correlated with NEAT1 in tumors. Functionally, knockdown of NEAT1 significantly inhibited cell proliferation and invasion and induced cell cycle arrest at the G0/G1 phase and apoptosis, whereas inhibition of miR-365 abolished the suppressive effect of NEAT1 knockdown on cellular processes. RGS20, a direct target of miR-365, could reverse the tumor suppressive role of miR-365 mimic by enhancing cell viability and motility. Moreover, the protein levels of RGS20, cyclin D1, E-cadherin, N-cadherin and vimentin could be regulated by the NEAT1/miR-365 axis. NEAT1 silencing also inhibited tumor growth in vivo. Collectively, we revealed that the NEAT1/miR-365/RGS20 axis may be a novel mechanism or therapeutic strategy for OSCC treatment.

Introduction

Oral squamous cell carcinoma (OSCC) is the most prevalent type of head and neck cancer and it accounts for nearly 90% of all oral cancer cases (1). Despite improvement in OSCC treatment over the past years, the 5-year survival rate of OSCC patients has not significantly improved (2). Therefore, identifying effective biomarkers and therapeutic targets is essential to acknowledge the molecular mechanisms underlying the progression of OSCC.

Among the dysregulated miRNAs, miR-365 was reported to be decreased in colon cancer, inhibited cell cycle progression and induced apoptosis (17). NKX2-1, a direct target of miR-365, attenuated the suppressive function of miR-365 on cell proliferation in lung cancer (18). In gastric cancer, activation of Akt decreased the expression of miR-365, consequently promoting cell growth by increasing the expression of cyclin D1 and CDC25A (19).

In the present study, we determined that the expression of NEAT1 was increased in OSCC and associated with tumor progression. Knockdown of NEAT1 inhibited cell proliferation and invasion and induced cell cycle arrest at the G0/G1 phase. Furthermore, we found that NEAT1 could act as a ceRNA of miR-365 and therefore regulate its target gene RGS20.

Materials and methods

Cell lines and clinical samples. OSCC cell lines (SCC-9, SCC-25, HN4, Tca-8113 and Cal-27) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA).
supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), and 100 µg/ml penicillin/streptomycin (BioLight, Shanghai, China). A human normal oral keratinocyte cell line (hNOK) was used as a control. All cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Thirty OSCC tissues and their adjacent non-tumor tissues were obtained from patients at the Department of Stomatology, General Hospital of Benxi Iron and Steel Co., Ltd. (Benxi, China), between 2010 and 2012. The present study was approved by the Research Ethics Committee of the General Hospital of Benxi Iron and Steel Group Co., Ltd., and written informed consents from patients were signed before surgery. None of the patients had a prior history of cancer or had received radiochemotherapy before surgery. All tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C until use. The clinicopathological characteristics of patients were summarized in Table I.

Quantitative real-time PCR (qPCR). Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). To assess NEAT1 and RGS20 expression, a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA) and SYBR Premix Ex Taq (Takara Biotechnology Co., Ltd., Dalian, China) were used for reverse transcription (RT) and qPCR, respectively. For the expression of miR-365, the TaqMan MicroRNA Reverse Transcription Kit and the TaqMan Universal Master Mix II (both from Applied Biosystems; Thermo Fisher Scientific, Inc.) were used for RT and qPCR, respectively. The results for NEAT1 and RGS20 were normalized to the expression of U6. The relative expression level of each gene was calculated and normalized using the 2^(-ΔΔCt) method.

Plasmid and oligonucleotide. The small interfering RNAs (siRNAs) specifically targeting NEAT1 (si-NEAT1 sense, 5'-GAGGGAUGAGGGUGAAGAA-3' and antisense, 5'-UUCUUCACCCUCAUCCCUC-3') and the negative control siRNA (si-NC) were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). For in vivo analysis, cells were transfected with 6 µg of sh-NEAT1 or the empty lentiviral vector, cultured with DMEM containing 20% FBS until use. The clinicopathological characteristics of patients were summarized in Table I.

Plasmid and oligonucleotide. The small interfering RNAs (siRNAs) specifically targeting NEAT1 (si-NEAT1 sense, 5'-GAGGGAUGAGGGUGAAGAA-3' and antisense, 5'-UUCUUCACCCUCAUCCCUC-3') and the negative control siRNA (si-NC) were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). For in vivo analysis, cells were transfected with 6 µg of sh-NEAT1 or the empty lentiviral vector, cultured with DMEM containing 20% FBS for 36 h. Lentiviral particles were harvested and used for infection. The target sequence of sh-NEAT1 was as follows: 5'-GCCATCAGCTTGATAAAATT-3'. The human NEAT1 gene was ligated into the pCDM8/PGM/CMV/MCS/RFP/Neo vector (Shanghai GenePharma Co., Ltd., Shanghai, China) and stable cell lines were generated by selection with G418 (Invitrogen; Thermo Fisher Scientific, Inc.). The generated lentiviruses were named sh-NEAT1 and the empty lentiviral vector (sh-Control) was used as a control. The miR-365 mimic, miR-365 inhibitor, mimic negative control (mim-NC), inhibitor negative control (inh-NC) sequences were obtained from Shanghai GenePharma Co., Ltd. Cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). To restore RGS20 expression, the Cal-27 cells were transfected with a pcDNA3.1-RGS20 plasmid (pcRGS20), which contained the coding sequences but lacked the 3'-UTR of RGS20. Cells transfected with the empty vector were used as a control and named pcDNA.

Cell proliferation analysis. Cells (1.5x10⁵/well) were plated in 96-well culture plates and cell viability was assessed every 24 h after transfection. MTT [5 mg/ml in phosphate-buffered saline (PBS); Sigma-Aldrich, St. Louis, MO, USA] was added to each well and the plates were incubated at 37°C. After 4 h, 150 µl dimethyl sulfoxide (DMSO) was added to each well. The absorbance was measured at 490 nm on a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Cell cycle and apoptosis analysis. At 48 h post-transfection, cells were harvested, washed with PBS, and fixed with 70% ethanol. Then the fixed cells were washed with PBS, centrifuged at 1,500 x g for 5 min and subsequently treated with RNase A (0.1 mg/ml) and propidium iodide (PI; 0.05 mg/ml) at 37°C for 30 min. The stained cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA). For the apoptosis assay, 48 h post-transfection, the cells were collected by trypsinization and washed twice with serum-containing medium. The cells were collected and resuspended in 1X Annexin V Binding buffer (Annexin V-FITC Apoptosis Detection kit; BD Pharmingen; BD Biosciences) at a concentration of 1x10⁶ cells/ml. Then, 5 µl of FITC Annexin V and 5 µl PI (BD Pharmingen; BD Biosciences) were added to 100 µl of the cell suspension. After incubation for 10 min at room temperature in the dark, 400 µl of binding buffer was added. Apoptosis was analyzed by flow cytometry (FACSCalibur; BD Biosciences) and the data were analyzed using CellQuest software (BD Biosciences).

Cell invasion assay. Cell invasion abilities were detected using Transwell chambers precoated with Matrigel (BD Biosciences). DMEM with 10% FBS was added to the lower chamber. OSCC cells were transfected, incubated, and then stained in serum-free DMEM overnight. Subsequently, they were resuspended (1x10⁶ cells) in serum-free medium, which was added to the upper chamber. Twenty-four hours later, the cells that had invaded to the lower surface of the membrane were fixed, stained and counted under an inverted microscope (Olympus, Tokyo, Japan) by counting five random fields.

Luciferase activity assay. The fragment from NEAT1 containing the predicted miR-365 binding site was amplified by PCR and cloned into a pmirGLO Dual-Luciferase Target Vector (Promega Corp., Madison, WI, USA) to form the NEAT1-MUT. Cells were co-transfected with either wild-type fragments or mutant fragments and miR-365 mimic or mim-NC using Lipofectamine 2000. A luciferase reporter assay was performed using the Dual-Luciferase Reporter Assay system (Promega Corp.).

The 3'-UTR of RGS20 containing the putative binding sites for miR-365 was amplified by PCR and cloned into the pGL3-luciferase reporter plasmid (Promega Corp.). Mutations in the miR-365-binding site of RGS20 3'-UTR were generated by the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Cells were co-transfected with miR-365 mimic (or mim-NC) and the reported vector with the wild-type (WT) or mutant (MUT) 3'-UTR of RGS20. Luciferase activity was assessed after incubation for 48 h at 37°C.
Western blot analysis. Cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) and the protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Equal amounts of protein were isolated using SDS-PAGE and then transferred to a polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Bedford, MA, USA). The membranes were blocked in 5% non-fat milk/TBST and incubated with primary antibodies. The primary antibodies RGS20 (1:500; cat. no. ab191500), cyclin D1 (1:1,000; cat. no. ab134175), E-cadherin (1:2,000; cat. no. ab15148), N-cadherin (1:2,000; cat. no. ab18203), vimentin (1:2,000; cat. no. ab24525) and β-actin (1:2,000; cat. no. ab8227) were purchased from Abcam (Cambridge, MA, USA). Subsequently, the membranes were incubated with goat anti-rabbit secondary antibodies (1:2,000; cat. no. ab150077) and the proteins were detected with ECL reagents (Pierce; Thermo Fisher Scientific, Rockford, IL, USA).

Statistical analysis. SPSS 16.0 software (IBM, Armonk, NY, USA) and GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, USA) were used for statistical analysis. The paired samples t-test was used to compare gene expression levels between OSCC and non-tumor controls. The overall survival of patients was analyzed by the Kaplan-Meier method. One-way ANOVA or Student's t-test was used for comparisons between the groups. P<0.05 was considered to indicate a statistically significant result.

Results

NEAT1 is overexpressed in OSCC cells and tissues. In order to know the relevance of NEAT1 in OSCC development, we assessed the endogenous levels of NEAT1 in OSCC cells. As shown in Fig. 1A, the expression of NEAT1 was significantly increased in OSCC cells compared to hNOK cells. Then, a qPCR assay was performed to evaluate the expression of NEAT1 in clinical samples. The expression of NEAT1 in OSCC tissues was significantly higher than that in matched non-tumor tissues (3.006±1.182 vs. 1.712±0.971, P=0.0004; Fig. 1B).

Table I. The expression levels of NEAT1 and miR-365 in subgroups of OSCC cases.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases, n=30</th>
<th>NEAT1 levels</th>
<th>P-value</th>
<th>miR-365 levels</th>
<th>P-value</th>
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<td></td>
<td></td>
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<tr>
<td>&lt;55</td>
<td>12</td>
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<td>0.657a</td>
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<td>≥55</td>
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<tr>
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<tr>
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<td>Gingiva</td>
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<td>2.517±0.767</td>
<td></td>
<td>0.621±0.357</td>
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*aMann-Whitney test; bKruskal-Wallis test; P<0.05 is indicated in bold. OSCC, oral squamous cell carcinoma; LNM, lymph node metastasis; never, no smoking history or has stopped smoking for >5 years; quit, has stopped smoking for <5 years.
Elevated NEAT1 is correlated with aggressive tumor phenotypes and poor prognosis. OSCC cases were classified into different subgroups such as sex (male vs. female) and TNM stage (I/II vs. III/IV). We determined that the expression levels of NEAT1 were significantly increased in cases with lymph node metastasis (P=0.009) and higher clinical stage (P=0.018; Table I), respectively. The median level of NEAT1 in tumors was used as a cut-off value to divide cases into two groups. Patients with high NEAT1 expression had poor survival when compared to patients with low NEAT1 expression (P=0.01; Fig. 1C).

NEAT1 negatively regulates miR-365 in OSCC. The exact function and underlying mechanism of NEAT1 in OSCC warranted further investigation. Using starBase 2.0 and RegRNA2.0, miR-365 was determined to potentially bind to NEAT1 (Fig. 1D), implying a possible interaction between miR-365 and NEAT1. Cal-27 and Tca-8113 cells were used for further analysis. We determined that the levels of miR-365 were significantly increased by si-NEAT1 transfection in both Cal-27 and Tca-8113 cells (Fig. 1E and F). The expression of NEAT1 was downregulated by miR-365 mimic, while it was upregulated by the miR-365 inhibitor (Fig. 1G and H). Co-transfection of miR-365 and NEAT1-WT significantly decreased the luciferase activity (Fig. 1I).

miR-365 was significantly downregulated in OSCC tissues compared to non-tumor tissues (0.622±0.364 vs. 0.819±0.428, P=0.0012; Fig. 1J). miR-365 was significantly downregulated in tumors of advanced stage (P=0.039, Table I). Cases were grouped in a low or high group according to the median level of miR-365. Patients with low expression of miR-365
appeared to have poor prognosis but without statistical significance (P=0.056; Fig. 1K). In addition, NEAT1 expression was negatively correlated with the expression of miR-365 in tumors (P=0.019, Pearson r=-0.423; Fig. 1L). These findings

Figure 2. miR-365 inhibitor attenuates the suppressive effect of knockdown of NEAT1 on cell growth and motility. (A) An MTT assay was conducted to evaluate the effect of the miR-365 inhibitor and si-NEAT1 on cell proliferation. (B) Knockdown of NEAT1 induced an increase in the percentage of cells at the G0/G1 phase and a decrease of cells at the S phase, a phenomenon that could be reversed by the miR-365 inhibitor. (C) Knockdown of NEAT1 contributed to cell apoptosis, whereas the miR-365 inhibitor had the opposite effect on cell apoptosis. (D) The number of invasive cells was increased by the miR-365 inhibitor, while it was reduced by si-NEAT1. *P<0.05, **P<0.01. si-NC, negative control siRNA; inh-NC, inhibitor negative control; si-NEAT1, specifically targeting NEAT1.
indicated that an interaction between NEAT1 and miR-365 may be involved in the development of OSCC.

**Inhibition of miR-365 attenuates the NEAT1 knockdown-induced inhibition of cellular processes.** To explore the effect of NEAT1 knockdown and miR-365 inhibition on cellular processes, we transfected OSCC cells with si-NEAT1 (or si-NC) and miR-365 inhibitor (or inh-NC). Knockdown of NEAT1 significantly reduced cell proliferation and abrogated the miR-365 inhibitor-induced increase of the cell proliferation rate (Fig. 2A). Flow cytometric analysis revealed that si-NEAT1 induced an increase in the percentage of cells at the G0/G1 phase and a reduction in the percentage of cells at the S phase, while the miR-365 inhibitor had an opposite effect on cell cycle distribution (Fig. 2B). Knockdown of NEAT1 induced cell apoptosis and abolished the miR-365 inhibitor-induced decrease of apoptotic cells (Fig. 2C). In addition, knockdown of NEAT1 could inhibit the invasive ability of cells, which was promoted by the miR-365 inhibitor (Fig. 2D). These results revealed that NEAT1 contributed to cell proliferation and invasion by negatively-mediated miR-365.

**RGS20 is a target of miR-365.** Numerous studies have revealed that lncRNAs could competitively suppress miRNAs by acting as ceRNAs, and ultimately regulate the expression of protein-coding genes. Thus, we searched for candidate genes of miR-365 using TargetScan, microRNA, miRDB and TargetMiner. Among the predicted targets, in the present study we focused on RGS20, considering the involvement of RGS20 in human cancers. In addition, by analyzing datasets from Oncomine, we determined that the mRNA levels of RGS20 were significantly upregulated in tongue squamous cell carcinoma (data not shown). All four bioinformatics tools revealed that the 3'-UTR of RGS20 mRNA has a potential binding site of miR-365 (Fig. 3A). Furthermore, miR-365 increased the number of apoptotic cells, which could be inhibited by RGS20 overexpression. (G) The miR-365 mimic abolished the increase of invasive cells induced by RGS20. *P<0.05, **P<0.01. UTR, untranslated region; OSCC, oral squamous cell carcinoma; WT, wild-type; MUT, mutant; mim-NC, mimic negative control.
In addition, overexpression of miR-365 significantly decreased the mRNA expression of RGS20 in OSCC cells (Fig. 3C).

**RGS20 is a functional target of miR-365**. To ascertain whether miR-365 performs its suppressive function through downregulation of RGS20, Cal-27 cells were co-transfected with mimic (or mim-NC) and pcRGS20 (or pcDNA). Cell proliferation was stimulated by overexpression of RGS20 and inhibited by the miR-365 mimic (Fig. 3D). Cell cycle analysis revealed that pcRGS20 transfected cells displayed a higher frequency of cells at the S phase and a lower frequency of cells at the G1 phase and ectopic expression of RGS20 reversed the miR-365-induced accumulation of G0/G1 phase cells (Fig. 3E). The apoptosis of Cal-27 cells was increased by the miR-365 mimic, while it was reduced by overexpression of RGS20 (Fig. 3F). Overexpression of RGS20 increased cell invasion (Fig. 3G), which was similar to the effect of the miR-365 inhibitor. RGS20 overexpression also significantly attenuated miR-365-induced inhibition on cellular invasion (Fig. 3G). These data indicated that miR-365 performs its tumor-suppressive function by regulating RGS20.

**Dysregulation of the NEAT1/miR-365/RGS20 axis is involved in epithelial-mesenchymal transition (EMT) and tumor growth**. Western blot analysis was performed to evaluate the effect of NEAT1/miR-365/RGS20 on the protein expression of cell cycle- and EMT-related markers. As shown in Fig. 4A, the protein expression of RGS20 was decreased by knockdown of NEAT1, while it was increased by the miR-365 inhibitor. Inhibition of NEAT1 abolished the miR-365 inhibitor-induced upregulation of RGS20 (Fig. 4A). Downregulation of NEAT1 led to an increase of E-cadherin and a reduction of cyclin D1, N-cadherin and vimentin, a phenomenon that could be reversed by the miR-365 inhibitor (Fig. 4A). Subsequently, the protein level of RGS20 could be inhibited by the miR-365 mimic (Fig. 4B), which was consistent with previous data shown in Fig. 3C. Restoration of RGS20 promoted the protein expression of E-cadherin (Fig. 4B).

To ascertain whether knockdown of NEAT1 inhibits tumor growth in vivo, Cal-27 cells (expressing sh-NEAT1 or sh-ctrl) were injected into the flanks of nude mice. The results indicated that the tumor volumes and weights formed by the sh-NEAT1 cells were markedly lower than those formed by the sh-ctrl cells (Fig. 4C and D). In addition, the tumors of sh-NEAT1-treated mice had a significantly low level of NEAT1, increased expression of miR-365, and downregulation of RGS20 (Fig. 4E-G). Furthermore, the protein levels of RGS20 were decreased in mouse tumors transfected with sh-NEAT1 compared to the control group (Fig. 4H).

**Discussion**

Numerous studies have shown that IncRNAs function as onco-genes or tumor-suppressor genes to regulate carcinogenesis, and that they can be used as diagnostic or prognostic markers (20). In the present study, the expression of NEAT1 was markedly increased in OSCC cells and tissues, and upregulation of NEAT1 was correlated with advanced stage and unfavorable
prognosis of OSCC patients. Similarly, high expression of NEAT1 was associated with metastasis and vaso-invasion in hepatocellular carcinoma (21). High NEAT1 was closely related to larger tumor size and independently associated with risk of death in glioma (22), as well as clinical pathologic grade in bladder cancer (23). Our results revealed the oncogenic role of NEAT1 in the development of OSCC. Certainly, further analysis based on a larger number of cases would provide more knowledge on the clinical relevance of NEAT1 in OSCC.

Functionally, knockdown of NEAT1 exerted a tumor-suppressive effect by inhibiting cell proliferation, cell cycle progression, and invasion in vitro and tumorigenesis in vivo, which was consistent with previous studies (10,23-25). NEAT1 could negatively regulate the expression of miR-365. miR-365 inhibition abrogated the inhibitory effect of NEAT1 knockdown. Several other miRNAs, including miR-377, miR-335, miR-107, miR-98 and miR-506, were identified to interact with NEAT1 in different types of cancers (12,14,26-28), suggesting that NEAT1 plays an oncogenic role in different types of cancer through the regulation of different miRNAs.

Our findings revealed that miR-365 suppressed cell proliferation and invasion and expanded on the knowledge of miR-365 as a tumor suppressor in OSCC. The inhibitory effect of miR-365 on tumorigenesis has also been reported in several studies (17-19,29). However, miR-365 displayed the opposite effect in cutaneous tumors by facilitating tumor growth (30). Thus, miR-365 exerts a tumor-suppressive or oncogenic function depending on its target genes. In the present study, RGS20 was identified as a direct target of miR-365 and overexpression of RGS20 impaired the miR-365-induced inhibition of cell growth and invasion. In addition, cyclin D1, CDC25A, WNT5A and ADAM10 were identified as targets of miR-365 and were correlated with miR-365-mediated cell growth and metastasis (19,29,31).

RGS20 was first reported to be overexpressed in metastatic melanomas (32). High expression of RGS20 indicated the progression and poor survival of triple-negative breast cancer (33). RGS20 facilitated cell aggregation, invasion and the expression of vimentin, but decreased the expression of E-cadherin (34). By gain-of-function approaches, we revealed similar results. RGS20 increased cell viability, motility and protein expression of cyclin D1, N-cadherin but decreased the protein level of E-cadherin, suggesting the oncogenic function of RGS20 in OSCC. The protein level of RGS20 was regulated by NEAT1/miR-365, suggesting that NEAT1 acted as a ceRNA of miR-365 and enhanced the expression of RGS20. Moreover, cell cycle- and EMT-related indicators were regulated by the NEAT1/miR-365/RGS20 pathway, supporting the regulatory effect of the NEAT1/miR-365/RGS20 axis on cell growth and metastasis in vitro. A previous study also demonstrated that NEAT1 is a regulator of EMT-related proteins in gastric cancer (35). Our findings revealed that RGS20, a direct target of miR-365, could mediate the biological effects that NEAT1 exerted.

In conclusion, we determined that upregulated NEAT1 was correlated with an aggressive tumor phenotype and an adverse prognosis in OSCC. NEAT1 promoted OSCC cell proliferation, cell cycle progression and invasion through the miR-365/RGS20 axis. These data provide new insights into the regulatory function of NEAT1/miR-365/RGS20 in the development of oral malignancy.

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Availability of data and materials
Not applicable.

Authors' contributions
GH conceived and designed the experiments. GH and XLW performed the experiments. XH analyzed the data. GH and XH wrote the manuscript. All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version to be published.

Ethics approval and consent to participate
The present study was approved by the Research Ethics Committee of the General Hospital of Benxi Iron and Steel Co., Ltd., and written informed consents from patients were signed before surgery. All animal procedures were in line with the guidelines of the Laboratory Animal Centre and were approved by the Ethics Committee of the General Hospital of Benxi Iron and Steel Co., Ltd.

Consent for publication
Not applicable.

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

References


