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The simultaneous miR-155-5p overexpression and miR-223-3p inhibition can activate pEMT in oral squamous cell carcinoma

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Abstract

Objective: This study aims to explore the effects of miR-223-3p and miR-155-5p on epithelial-mesenchymal transition (EMT) and migration in oral squamous cell carcinoma (OSCC). Methodology: EMT markers (E-cadherin, N-cadherin, P120 catenin (P120ctn), and vimentin) expression was determined by qRT-PCR and western blot analysis in SCC-9 cells which overexpress miR-155-5p and/or not express miR-223-3p. Scratch assays and Transwell migration assays were conducted to evaluate cell migration ability. Results: When miR-223-3p was inhibited in OSCC cells, P120ctn and E-cadherin mRNA levels were dramatically downregulated ($P<0.05$), while N-cadherin levels were significantly upregulated, and the migration ability of OSCC cells increased. The overexpression of miR-155-5p in OSCC cells upregulated miR-223-3p significantly (34-fold) compared to the control group. It also led to significant downregulation of the mRNA of P120ctn and E-cadherin and significant upregulation of the mRNA of N-cadherin and Vimentin ($P<0.05$). Meanwhile, the migratory ability of OSCC cells significantly increased. When miR-155-5p was overexpressed while miR-223-3p was inhibited, the highest expression of E-cadherin and P120ctn mRNA and the lowest expression of N-cadherin($P<0.05$) was observed. Simultaneously, tumor cell migration was significantly facilitated. Conclusion: miR-223-3p inhibits the migration of OSCC cells, while miR-155-5p can elevate the miR-223-3p mRNA expression. The simultaneous miR-155-5p overexpression and miR-223-3p inhibition can activate pEMT, increasing OSCC migration *in vitro*. This provides a novel approach and potential target for the effective treatment of OSCC.

Keywords: miR-223-3p. miR-155-5p. Migration. Oral squamous cell carcinoma. EMT

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Introduction

Oral squamous cell carcinoma (OSCC) poses a significant public health concern, representing 80% of oral and maxillofacial cancers, with an approximate 50% 5-year survival rate.[1] Epithelial-mesenchymal transition (EMT), a crucial developmental process[2] plays a significant role in the metastasis of various cancers, including those of the lung, breast, ovary, liver, stomach, and colon.[3] It refers to the biological process through which epithelial cells transform into cells with a mesenchymal phenotype. In OSCC, EMT's effect on tumor recurrence and metastasis is a significant prognostic factor.[4,5]

During EMT, there is a downregulation of epithelial markers, such as E-cadherin (E-cad), α-catenin, and β-catenin, and an upregulation of mesenchymal markers including N-cadherin (N-cad) and vimentin (Vim).[6] This process leads to aggressive characteristics in epithelial cells, such as reduced cell adhesion, loss of tight junctions, altered cell polarity, and increased mobility. However, many studies have shown that cancer cells can stably acquire one or more partial EMT (pEMT) phenotypes displaying tumor-migration potential, and that they can exhibit a mixture of epithelial and mesenchymal characteristics at the molecular and/or morphological level.[7,8,9]

E-cad plays a crucial role in maintaining cell-cell junctions[10] and its downregulation is a hallmark of EMT.[11] Meanwhile, it has been well-documented that P120 catenin (P120ctn) interacts with E-cad, regulating its stability and expression.[12,13,14] Moreover, reduced P120ctn expression is associated with downregulated E-cad, promoting OSCC invasion and migration.[15] Additionally, N-cad promotes tumor cell invasion and metastasis by indirectly affecting the synthesis and transcription of E-cad.[16] Accumulating evidence has revealed that in OSCC with lymph node metastasis, N-cad expression is increased, while E-cad expression is reduced, suggesting a cadherin transformation from E-cad to N-cad in these tissues.[17,18] Furthermore, Vim is upregulated in various epithelial tumors, including OSCC, and is associated with tumor invasion and poor prognosis.[19,20] In addition, in OSCC, Vim expression is elevated in poorly differentiated and lymph node metastatic tumors, indicating the acquisition of an EMT phenotype.[16,17,18,21]

Intriguingly, recent evidence has uncovered that the human genome dedicates a relatively small proportion of its transcribed RNA to protein coding, while the majority consists of non-coding RNAs, such as microRNAs (miRNAs), small interfering RNAs, and long non-coding RNAs.[22] Despite their low abundance, endogenous miRNAs regulate 30% of genes in the human genome by binding to the target mRNA's 3' untranslated region (3'-UTR), leading to translation inhibition or degradation,[23] playing critical roles in tumor formation,[24] occurrence, and development.[25]

Recent studies attached importance to the role of miR-223-3p and miR-155-5p in various cancers. The overexpression of miR-223-3p promotes colon cancer progression through the downregulation of P120ctn and E-cad, and the upregulation of Vim, leading to increased cell proliferation, migration, and invasion.[26,27] Conversely, overexpressing miR-233-3p in nasopharyngeal carcinoma cells suppressed cell proliferation and migration.[28] The role of miR-223-3p in OSCC via EMT markers such as P120ctn, E-cad, N-cad, and Vim remains to be confirmed. Moreover, miR-155-5p, highly expressed in various tumors,[29,30] including OSCC, is associated with metastasis, poor prognosis, and EMT progression.[31,32] Given their inclusion in the same miRNA subnetwork across 2532 solid cancers,[33] both miR-155-5p and miR-223-3p may contribute to tumor pathogenesis.

The experiments described here provide a theoretical basis for OSCC-targeted therapy by exploring the correlation between miR-223-3p and miR-155-5p and EMT markers and their mechanism in the migration of oral squamous cell carcinoma cells.

Methodology

Cell culture and transfection

Human oral squamous cell carcinoma cell strain SCC-9 was purchased from Shanghai Fuheng Biotechnology Co., Ltd. SCC-9 cells were maintained in DMEM/F-12 [HAM]1:1 medium containing 10% fetal bovine serum and 0.4 μg/ml hydrocortisone at 37°C in humidified air with 5% CO_2.

SCC-9 cells were divided into 4 groups based on the nucleotide transfected: negative control group (NC), miR-223-3p inhibitor (miR-223-3p(-)), miR-223-3p inhibitor/miR-155-5p mimics group (miR-223-3p(-)/miR-155-5p(+), and miR-155-5p mimics group (miR-155-5p(+)). Transfection was conducted using Lipofectamine 2000 (Invitrogen, USA) according to

the manufacturer's instructions.

Extraction of microRNA and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA and miRNAs were isolated using the miRcute miRNA Isolation Kit (DP501, Tiagen, China) according to the manufacturer's instructions. The concentration and purity of the extracted mRNA and miRNAs were determined using a NanoDrop One ultra-microphotometer (ThermoFisher, USA). The complementary DNA (cDNA) was synthesized using a SuperScript™ IV First-Strand Synthesis System (for microRNAs, #18091050, ThermoFisher Scientific) or a NovoScript®Plus All-in-one 1st Strand cDNA Synthesis SuperMix (for RNAs, E047-01B, Novoprotein) according to the manufacturer's instructions. The resulting cDNAs were amplified on an A600 Super Gradient Thermo Cycler (LongGene, Hangzhou, China). The relative expression levels of miR-223-3p, E-cad, N-cad, P120ctn, and Vim, were calculated using the $2^{-\triangle\triangle Ct}$ method. U6 and GAPDH served as the internal references for microRNAs and RNAs, respectively. All primers used in this study (Figure 1) were synthesized by Borui Biotechnology Col, Ltd (Xiamen, China).

Western blot analysis

SCC-9 cells were harvested and solubilized using RIPA lysis buffer supplemented with protease inhibitors (P1045, Beyotime, 1:50 dilution) and EDTA (1:50 dilution), followed by centrifugation (4°C, 10,000 rpm, 5 minutes). The cleared cell lysate was collected and protein concentration was determined using a Bradford protein concentration determination kit (P0006, Beyotime).[34] An equal amount of total cell lysate was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Subsequently, the membranes were blocked with 5% skim milk and incubated with primary antibodies overnight at 4°C (Figure 2). Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The enhanced chemiluminescence reagent was used to develop the membranes, and images were captured using a ChemiDoc imaging system (BioRad, USA) and the gray scale of the bands were analyzed with ImageJ 1.45 s software (National Institutes of Health, USA) to reflect the relative protein expression levels.

Cell migration assays

For scratch assays, SCC-9 cells under the indicated conditions were cultured in six-well plates until confluent. Scratches were then made using a 10 µL pipette tip. After the detached cells were washed off, the adherent cells were photographed under a microscope at the beginning (0 h) and the end (18 h) of the experiment, capturing images from five random

	Forward primer (5'-3')	Reverse primer (5'-3')
miR-223-3p	TCGGCAGGTGTCAGTTTGTC	CTCAACTGGTGTCGTGGAGT
U6	CTCGCTTCGGCAGCACATATACT	ACGCTTCACGAATTTGCGTGTC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
E-cad	CGAGAGCTACACGTTCACGG	GGGTGTCGAGGGAAAAATAGG
N-cad	TGCGGTACAGTGTAACTGGG	GAAACCGGGCTATCTGCTCG
Vim	AGTCCACTGAGTACCGGAGAC	CATTTCACGCATCTGGCGTTC
P120ctn	GTGACAACACGGACAGTACAG	TTCTTGCGGAAATCACGACCC

Figure 1- Sequences of primers

Antibody	Manufacturer	Catalog Number	Dilution
GAPDH	Abcam	ab181602	1: 10,000
Delta 1 catenin	Abcam	ab92514	1: 1,000
E-cadherin	Cell signaling Technology	2,40E+11	1: 1,000
N-cadherin	Invitrogen	MA5-32088	1: 1,000
Vimentin	Invitrogen	MA5-35320	1: 1,000
Goat-anti-Rabbit IgG	Lablead	S0101	1: 5,000
Goat anti-Mouse IgG	Lablead	S0100	1: 5,000

Figure 2- Antibodies used in this study

fields. Wound closure was reflected by the closure rate (0 h scratch width – 18 h scratch width)/0 h scratch width × 100%.

For the transwell migration assay, 100 µL of serum-free medium containing 1.5×10^5 SCC-9 cells under the indicated conditions were loaded into each insert of the upper chamber. Then, the upper chamber was placed into 12-well plates containing 200 µL of normal culture medium. After 18 hours, un-migrated cells were removed with a cotton swab and the migrated cells were washed with PBS and stained with 0.1% crystal violet (10 min, at room temperature). Subsequently, the migrated cells were photographed and counted in six random fields of each well.

Statistical analysis

All results were analyzed using the SPSS software (version 23.0, IBM). The data are expressed as means ± standard deviation (means ± SD), and all graphs presenting means ± SD represent data from at least 3 independent experiments. Pairwise comparisons were made using the t-test, and one-way ANOVA was performed for multiple comparisons, while LSD-t test was used for pairwise comparisons between groups. The two-sided test level was $\alpha=0.05$, and $P<0.05$ indicated that the difference was statistically significant.

Results

Effects of miR-155-5p on miR-223-3p expression in OSCC

SCC-9 cells were transfected with miR-223-3p inhibitor and miR-155-5p mimic, either separately or together. miR-223-3p and miR-155-5p expression was subsequently determined in the cells using RT-PCR analysis. We found that miR-223-3p expression was significantly elevated when the miR-155-5p mimic was transfected, and it decreased to 70% with miR-223-3p inhibition transfection. Remarkably, the suppressive effects of the miR-223-3p inhibitor were neutralized by miR-155-5p overexpression, as reflected by the 34-fold increase in miR-223-3p expression upon miR-155-5p mimic transfection. Meanwhile, miR-155-5p overexpression dramatically increased (184-fold) miR-223-3p ($P<0.05$) production. Together, these findings suggest a regulatory role of miR-155-5p on miR-223-3p expression in OSCC.

Regulation of EMT markers by miR-223-3p inhibitor and miR-155-5p mimic in OSCC cells

miR-223-3p is known to exert an inhibitory effect on EMT in nasopharyngeal carcinoma cells.[28] Thus, we examined whether this regulatory effect could be detected in OSCC. In SCC-9 cells, inhibiting miR-223-3p led to significant decreases in the expressions of E-cad and P120ctn, along with a significant increase in Vim and N-cad, compared to the control group (Figure 3A, $P<0.05$). Our data demonstrated that overexpression of miR-155-5p resulted in miR-223-3p upregulation. By qRT-PCR analysis, following the overexpression of miR-155-5p, the expression of E-cad and P120ctn, which were initially suppressed by the inhibition of miR-223-3p, exhibited an increase ($P<0.05$). However, miR-223-3p(-)/miR-155-5p(+) showed the highest expression of E-cad and P120ctn mRNA and the lowest expression of N-cad ($P<0.05$). To further validate our findings, we performed western blot analysis to assess the protein levels of the EMT markers. Surprisingly, despite a significant difference observed at the transcriptional level, the variations of the EMT markers at the protein level were marginal ($P>0.05$, Figure 3B and 3C).

Effects of transfection of miR-223-3p inhibitor and miR-155-5p mimic respectively or simultaneously on SCC-9 cell migration

We further investigated the impact of miR-223-3p and miR-155-5p on OSCC cells. Considering their influence on EMT markers, we reasoned that miR-223-3p and miR-155-5p might regulate the mobility of OSCC cells. To test this hypothesis, cell migration assays were conducted using SCC-9 cells transfected with or without altered miR-223-3p and/or miR-155-5p. Scratch assays revealed that inhibiting miR-223-3p facilitated wound closure compared to the control group (20.00% vs. 9.75%, $P<0.01$). In addition, overexpressing miR-155-5p also promoted cell mobility as reflected by enhanced wound closure compared to the control group (19.50% vs. 9.75%, $P<0.05$). Cells in the miR-223-3p(-)/miR-155-5p(+) showed the highest migratory capability (28.75% vs. 16.7%, $P<0.05$) (Figure 4A).

Meanwhile, transwell migration assays revealed comparable results. We observed more migrated SCC-9 cells from miR-223-3p(-) group and miR-155-5p(+) group compared to the negative control group ($P<0.001$). Remarkably, the cells exhibited the highest migratory capacity when miR-155-5p mimic and miR-

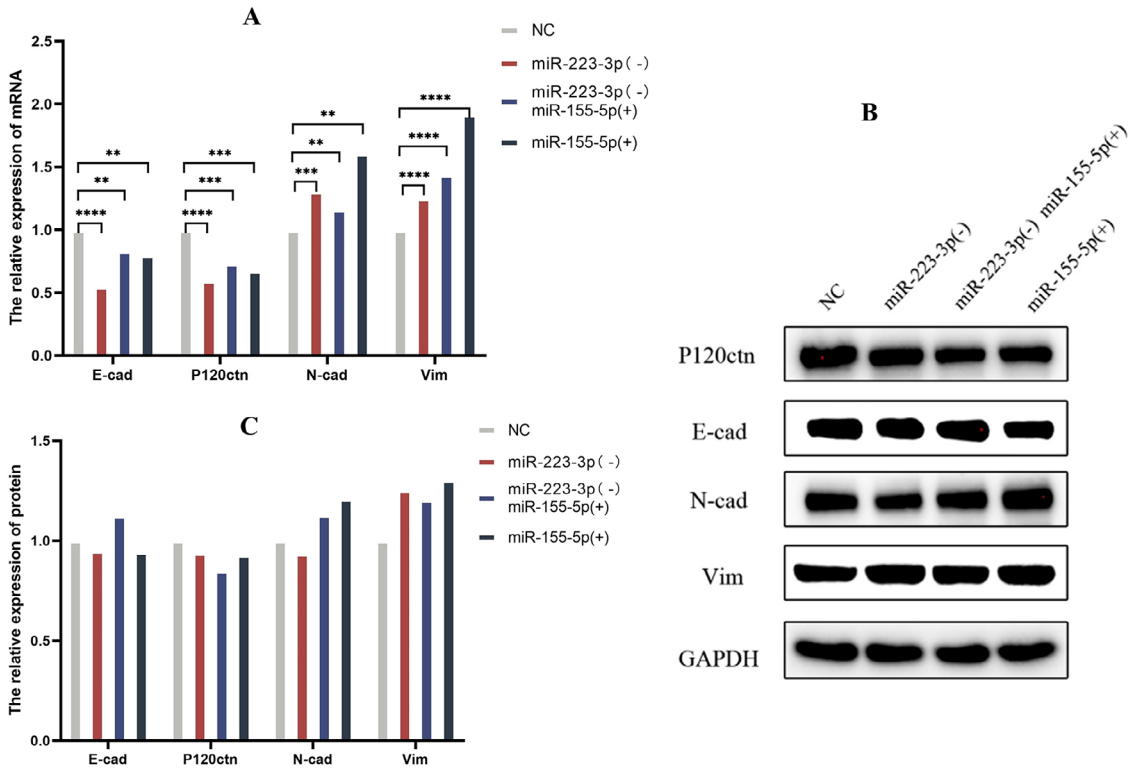

Figure 3- Effects of miR-223-3p and miR-155-5p on the expression of EMT markers in OSCC cells. (A) Results of RT-PCR showing the relative expression levels of EMT markers in SCC-9 cells under indicated conditions. (B) Representative images of western blot analysis showing the expression levels of EMT markers in SCC-9 cells under the indicated conditions. (C) Results of western blotting showing the relative expression levels of EMT markers in SCC-9 cells under the indicated conditions

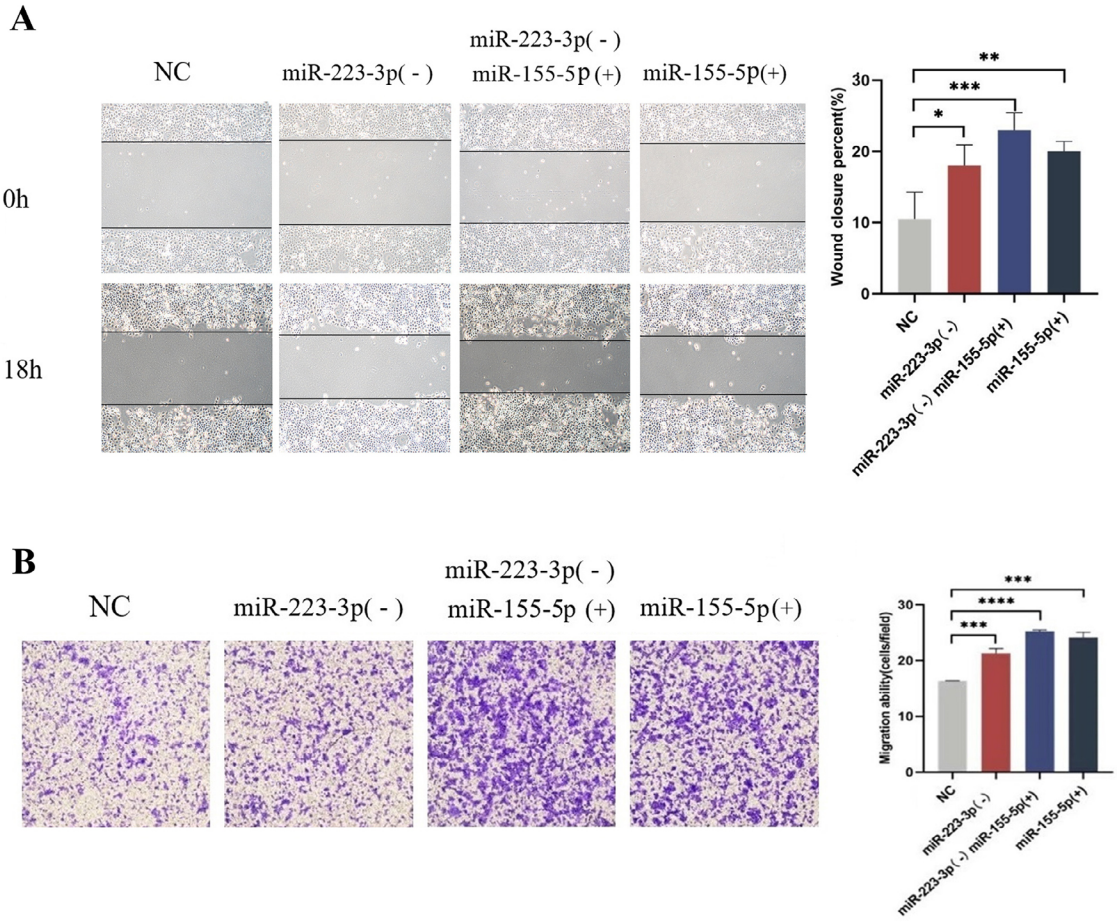

Figure 4- Both miR-223-3p and miR-155-5p are involved in SCC-9 cell migration. Representative images of (A) scratch assay and (B) transwell migration assay demonstrating the migration of SCC-9 cells under the indicated conditions

223-3p inhibitor were co-transfected ($P<0.0001$) (Figure 4B).

Discussion

OSCC accounts for 95% of head and neck tumors, with over 300,000 cases annually,[35] making it a major public health concern.[1] MicroRNAs play a regulatory role in diseases, including OSCC,[36] by regulating protein-coding genes. miR-223-3p is downregulated in OSCC tissues,[37] and is involved in tumor proliferation and migration by targeting FBXW7.[38] These data strongly suggest that miR-223-3p is a multifunctional regulatory miRNA involved in the pathogenesis of OSCC in humans.

In our study, inhibiting miR-223-3p promoted EMT and cell mobility in OSCC cells, as evidenced by decreased epithelial markers (E-cad and P120ctn) and increased mesenchymal markers (Vim and N-cad). This aligns with previous studies showing lower miR-223-3p expression in OSCC patients.[37] Upregulation of miR-223-3p in OSCC cells inhibits cell functions, such as proliferation, migration, and survival, by targeting SHOX2[37] or NLRP3,[39] leading to the inhibition of OSCC invasion. Separate studies in gingival cancer also found that miR-223 inhibits proliferation and induces apoptosis.[40] miR-223-3p had a diametrically opposite effect on EMT marker expression in colon cancer, probably because in colon cancer dual luciferase reporter assays showed that miR-223-3p directly targeted P120ctn and downregulated P120ctn expression,[41] whereas in SCC-9 miR-223-3p may also target other genes, resulting in upregulation of P120ctn.

Similarly, in our study, overexpression of miR-155-5p decreased E-cad and P120ctn while increasing N-cad and Vim, consistent with promotion of EMT and an invasive OSCC phenotype. This finding is consistent with that of Wu et al., showing that miR-155-5p enhances OSCC invasion and metastasis through EMT regulation.[42] Given the parallels between miR-223-3p inhibition and miR-155-5p overexpression, we speculate that these four EMT markers are co-regulated downstream targets in SCC-9 cells.

Notably, miR-155-5p positively regulated miR-223-3p, with a 184-fold increase in the miR-155-5p(+) group and 35-fold increase in the miR-223-3p(-)/miR-155-5p(+) group. This reveals an interacting miRNA network that cooperatively regulates EMT and the invasive phenotype. Indeed, prior studies found miRNA-miRNA interactions influence tumor growth and metastasis by regulating the expression of miRNA and mRNA.[43,44,45]

Surprisingly, despite this upregulation of the tumor suppressive miR-223-3p, migration was not further suppressed by miR-155-5p overexpression. Instead, significant migration was still observed, indicating that miR-155-5p's dominant pro-invasive effect overwhelms miR-223-3p's anti-migration influence. This reveals miR-155-5p likely has a greater functional influence on cell migration, even though it positively regulates miR-223-3p expression. miR-155-5p appeared to have a greater influence on tumor cell migration.

Moreover, cells in the miR-223-3p(-)/miR-155-5p(+) showed the highest migratory capability with the highest mRNA expression level of E-cad and p120ctn and the lowest N-cad expression.[46] EMT often associates with an almost complete loss of membranous localization of E-cad, i.e., a "cadherin switch" from E-cad(epithelial marker) to N-cad(mesenchymal marker).[47] Recent findings suggest that EMT and MET are not all-or-none responses, i.e., switching between purely epithelial and purely mesenchymal phenotypes[48] but rather multi-state processes called "partial EMT," which can lead to collective migration. The upregulated expression of E-cad supports a special, "tether-mediated" mode of collective migration, that enhances both stromal infiltration capacity and the efficient formation of metastases.[49] The lowest expression of N-cad[46] may be attributed to that it is a late EMT marker expressed only after full EMT.

The discrepancy between mRNA and protein expression of EMT markers in SSC-9 cells, especially when miR-223-3p inhibitor and miR-155-5p mimic were co-transfected, indicates a need for further research to better understand the communication between these two miRNAs in OSCC.

The current study has some limitations. While providing valuable insights, the current study suggests the need for further investigation. Though we focused the discussion on one or two lead possibilities based on prior literature, there may be multiple mechanisms underlying the observed phenotypes. Follow-up analyses are warranted to explore alternative explanations. Additionally, as only the SCC-9 cell line was employed here, examining other oral cancer

cell models would strengthen confidence in the findings and account for potential cell-specific effects. Expanding the experimental approaches could yield deeper mechanistic clarity while reinforcing the key conclusions made.

In summary, our investigation revealed the functional roles and influencing factors of miR-223-3p and miR-155-5p in OSCC. We established that miR-223-3p acts as a negative regulator in the development of OSCC and is closely related to EMT. The simultaneous miR-155-5p overexpression and miR-223-3p inhibition can activate pEMT increasing OSCC migration. Currently, few studies have examined the crosstalk between miR-223-3p and miR-155-5p, warranting further investigation to elucidate the primary regulatory mechanism of miR-155-5p on miR-223-3p. Such insights may pave the way for novel targeted therapies for OSCC.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors'contributions

Zhou, Ruiman: Conceptualization (Equal); Methodology (Equal); Writing - review & editing (Equal). **Chen, Zhong:** Project administration (Equal); Writing - original draft (Equal). **Cai, Yihuang:** Conceptualization (Equal); Methodology (Equal); Writing - review & editing (Equal). **Zhang, Huilian:** Conceptualization (Equal); Methodology (Equal); Writing - original draft (Equal). **Mao, Shunjie:** Formal analysis (Equal); Writing - review & editing (Equal). **Zhuang, Yunan:** Writing - review & editing (Equal). **Zheng, Jiacheng:** Writing - review & editing (Equal)

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