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Original Article

miR-376a/Neuropilin-1 axis mediates the cancer stemness in oral squamous cell carcinoma-an in vitro study

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KEYWORDS
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Abstract *Background/purpose:* The incidence of oral cancer has been steadily increased over the years. Recent researches indicated that exploring oral cancer stem cells (CSCs) characterized by self-renewal, pluripotency, and aggressiveness have emerged as a promising strategy for predicting oral squamous cell carcinoma (OSCC) recurrence and metastasis. Previous studies have demonstrated that microRNAs regulate cancer stemness. However, the mechanisms that miR-376a/neuropilin-1 (NRP1) axis influences CSC traits have not yet been fully understood. Therefore, an in-depth investigation was conducted in this study.
Materials and methods: miR-376a expression in CSCs derived from OSCC cell line SAS was quantified by quantitative real-time polymerase chain reaction. Aldehyde dehydrogenase 1 (ALDH1) activity and CD44 expression were assessed via flow cytometry. The CSC phenotype was characterized through self-renewal, migration, and colony formation assays. A luciferase reporter assay was used to confirm the direct interaction between miR-376a and NRP1.
Results: We found that miR-376a expression was downregulated in SAS-CSCs. Overexpression of miR-376a significantly reduced several CSC phenotypes including ALDH1 activity, CD44 expression, migration, and colony-forming abilities, respectively ($P < 0.05$). In addition, a luciferase reporter assay substantiated the direct binding of miR-376a to NRP1 ($P < 0.05$). Moreover, NRP1 overexpression was found to reverse miR-376a-induced the inhibition of migration and self-renewal, respectively ($P < 0.05$).
Conclusion: Within the limitations of our findings, miR-376a/NRP1 axis may play a crucial role in stemness of OSCC. Targeting this pathway could represent a promising strategy to inhibit OSCC progression.

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Introduction

According to global cancer statistics, oral cancer ranks as the sixth most common cancer worldwide.[1] In Taiwan, the majority of oral squamous cell carcinoma (OSCC) occur in tongue and buccal mucosa.[2] Despite the availability of various treatment options such as surgical resection, chemotherapy, and radiotherapy, five-year survival rate still remains approximately 53.9 %.[2] In addition, OSCC continues to be characterized by rapid progression, lymphatic metastasis, and poor prognosis.[3] One of the latest strategies for assessing the potential of OSCC recurrence is the study of cancer stem cells (CSCs).[4—6]

CSCs are a subpopulation of tumor cells that exist in low proportions within tumor tissue, typically comprising only 0.01—2 % of total tumor mass.[7] CSCs, known for their self-renewal, pluripotency, and aggressiveness, are thought to drive cancer relapse and metastasis.[8] Oral CSCs can be identified by the detection of various markers such as CD44, CD133, and aldehyde dehydrogenase 1 (ALDH1).[9] CD44 is a glycoprotein involved in intercellular communication, cell adhesion, and cell migration. $CD44^+$ population of oral cancer cells have been proven to possess the properties of CSCs.[10] ALDH1, a well-established marker of CSCs, belongs to the ALDH enzyme family involved in aldehyde oxidation and the conversion of retinol to retinoic acid during early stem cell differentiation. The expression of ALDH1 was shown to exhibit a strong correlation with tumor malignancy and the self-renewal capacity of stem cells across diverse tumor types, including OSCC.[11]

An increasing number of studies have revealed that non-coding RNAs possess the ability to regulate various physiological functions and even cancer stemness.[12,13] Non-coding RNAs can be broadly classified into two main categories: short non-coding RNAs (such as microRNAs, miR), which are 21—23 nucleotides in length, and long non-coding RNAs (lncRNAs), which are longer than 200 nucleotides.[14] Over past few decades, numerous miRNAs have been identified to be associated with the regulation of oral cancer stemness. For example, miR-21 plays a crucial role in maintaining stem cell properties and regulating CSC characteristics such as migration and invasion.[15] miR-520b has diverse activities in modulating anti-malignant properties including the enhancement of cellular sensitivity to radiation and chemotherapy, the restriction of cell motility, and the limitation of CSC formation.[16] In addition, miR-let-7a was found to suppress chemoresistance and tumorigenicity in head and neck cancer by inhibiting stem cell properties.[17] Therefore, continuous exploration of unknown miRNAs for the regulation of cancer stemness will help us gain a better understanding of how to develop the effective treatment strategies for OSCC.

Situated on human chromosome 14q32, miR-376a has been implicated in multiple cancer-related processes. It has been shown that dysregulation of miR-376a affects proliferation, aggressiveness, and apoptosis in various types of cancer such as glioblastoma,[18] melanoma,[19] hepatocellular carcinoma,[20] lung adenocarcinoma,[21] and metastatic prostate cancer.[22] In melanoma, insulin growth factor 1 receptor (IGF1R) was found to be a direct target of miR-376a.[19] A previous report has demonstrated that IGF1R signaling was implicated in the induction of Nanog expression and promotion of cancer stemness in lung cancer.[23] Yes-associated protein 1 (YAP1) has been identified as another potential target of miR-376a that can regulate glioma cell proliferation and promote cell apoptosis.[24] One of the latest studies has revealed that YAP drives the malignant reprogramming of oral epithelial stem cells. In addition, YAP-mediated tumor-initiating cell programs involve in the activation of oncogenic transcriptional networks and mTOR signaling.[25] Moreover, overexpression of miR-376a has been demonstrated to mitigate the stemness of triple-negative breast cancer cells as evidenced by the reduced expression of stemness markers, spheroid formation capacity, and ALDH1 activity, possibly through the regulation of neuropilin-1 (NRP-1).[26]

These studies suggest that miR-376a may serve a key function in the modulation of cancer stemness. However, the significance of miR-376a in oral CSCs has not yet been investigated. Herein, the aims of this in vitro study was to explore the functional role of miR-376a in regulating the stemness in OSCC and its putative target involved in this process.

Materials and methods

OSCC cell culture

The OSCC cell line SAS was cultured following the established protocols.[27] The expression of $ALDH1^+$ and $CD44^+$ SAS-CSCs were isolated by using the ALDEFLUOR assay kit (StemCell Technologies Inc., Vancouver, WA, USA) and sorted via FACSAria II cell sorting (BD Biosciences, New York, NJ, USA), as previously detailed.[28]

Sphere formation assay

Cells were dissociated and cultured as tumor spheres in modified DMEM/F-12 supplemented with N2 (R&D Systems, Minneapolis, MN, USA), 10 ng/mL epidermal growth factor (Invitrogen Life Technologies, Carlsbad, CA, USA), 10 ng/mL basic fibroblast growth factor (Invitrogen Life Technologies), and penicillin/streptomycin. A density of 10^3 live

cells per low-attachment six-well plate (Corning Inc., Corning, NY, USA) was used and the medium was changed every other day. Tumor sphere formation was observed after approximately two weeks. For the evaluation of self-renewal ability, single cells were obtained from accutase-treated spheroids, and the cell density of each passage was set at 1000 cells/mL in serum-free medium.[28] **Quantitative real-time PCR (qRT-PCR)**.

Total RNA was prepared from cells using Trizol reagent according to the manufacturer's protocol (Invitrogen Life Technologies). mRNAs were reverse-transcribed using the Superscript III first-strand synthesis system for RT-PCR (Invitrogen Life Technologies). qRT-PCR reactions on the resulting cDNAs were performed on an ABI StepOne™ Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) according to our previous study.[28]

Western blot analysis

The Western blot analysis was executed as per the previously described protocols.[29] The primary antibodies utilized were those specific to NRP1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Reporter construction and assay

The NRP1 3′UTR sequence was inserted into the pMIR-REPORT vector to create a wild-type reporter (WT). Briefly, a mutant reporter (MUT) was generated by altering the miR-376a binding site within the WT construct. Reporter activity was determined by measuring firefly luciferase activity normalized to transfection efficiency.[30] Cells were transfected using BioRad's Transfectin lipid reagent (Bio-Rad Laboratories, Marnes-la-Coquette, France).

Transwell migration assay

$1x10^5$ cells were seeded in the upper chamber of a transwell insert serum-free medium. Medium supplemented with 10 % FBS in the lower chamber served as a chemoattractant. After 24 h, migrated cells on the lower membrane surface were stained with crystal violet and counted in five random fields.[30]

Colony-formation assay

Six-well plates were prepared with a 0.6 % agar bottom layer and a 0.3 % agar top layer containing 2 x 10^4 cells. After four weeks of incubation at 37 °C, colonies were stained with 0.005 % crystal violet and counted. Colonies with a diameter of 100 μm or greater were counted in five random fields per well, totaling 15 fields across triplicate experiments.[31]

Statistical analysis

All data are expressed as the mean ± standard deviation (SD) of triplicate analyses. Student's t-test and One way ANOVA were employed to assess the statistical significance of differences between groups. $P < 0.05$ will be considered statistically significant.

Results

To assess whether miR-376a was aberrantly expressed in OSCC cell line SAS, we initiated this study by isolating ALDH1$^+$ and CD44$^+$ SAS-CSCs. Subsequently, we conducted a comparative analysis between the isolated SAS-CSCs and SAS cells. The results showed that miR-376a was down-regulated in both ALDH1$^+$ SAS-CSCs (Fig. 1A) and CD44$^+$ SAS-CSCs (Fig. 1B). In line with this finding, we showed that SAS cells transfected with miR-376a mimics exhibited lower ALDH1 activity and CD44 subpopulation. Specifically, ALDH1$^+$ cells decreased from 28.3 % to 6.2 % and CD44$^+$ cells decreased from 59.8 % to 7.1 %, respectively, following miR-376a overexpression (Fig. 2A and B). Taken together, these results suggest that miR-376a may function as a tumor suppressor in OSCC by attenuating its cancer stemness.

In an effort to examine this hypothesis, we analyzed several CSC characteristics in addition to stemness marker expression. Given that CSCs often exhibit a more malignant phenotype, we assessed the migratory capacity following miR-376a overexpression. The results showed the increased miR-376a expression resulted in approximately 60 % reduction in cell migration as compared with the control (Fig. 3A). In addition, we measured the colony-forming units to assess the proliferative capacity and tumorigenicity surrogates. As shown in Fig. 3B, cells with ectopic

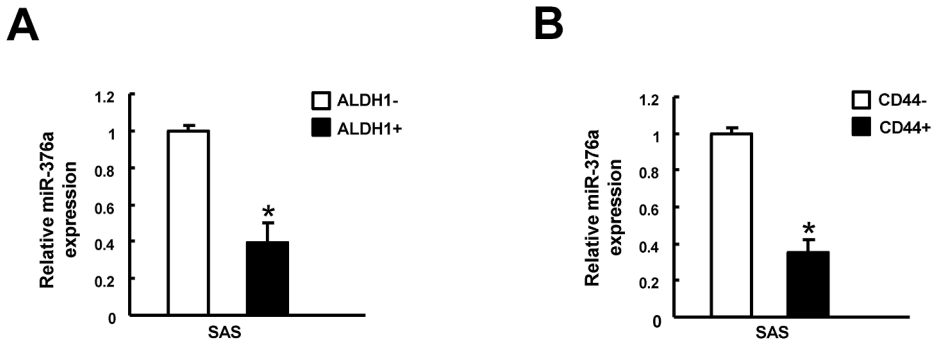

Figure 1 miR-376a is downregulated in oral cancer stem cells derived from SAS cell line. Results from quantitative real-time polymerase chain reaction showed that miR-376a was downregulated in (A) ALDH1$^+$ SAS cancer stem cells and (B) CD44$^+$ SAS cancer stem cells. *$P < 0.05$ compared to ALDH1$^-$ or CD44$^-$ non-cancer stem cells cells.

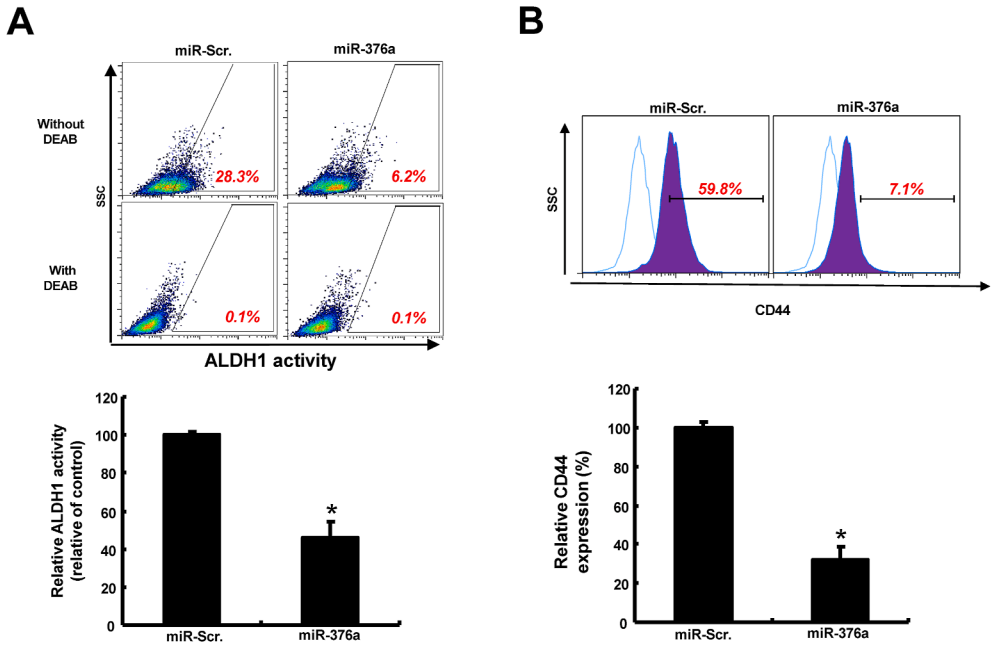

Figure 2 Overexpression of miR-376a inhibits ALDH1 activity and relative CD44^{+} expression in SAS cells. (A) Overexpression of miR-376a in SAS cancer stem cells reduced the Aldehyde dehydrogenase 1 (ALDH1) activity using flow-cytometry. (B) Upregulation of miR-376a attenuated the percentage of cells expressing CD44^{+}. *$P < 0.05$ compared to miR-Scr. miR-Scr: miR-Scranble; DEAB: N, N-diethylaminobenzaldehyde.

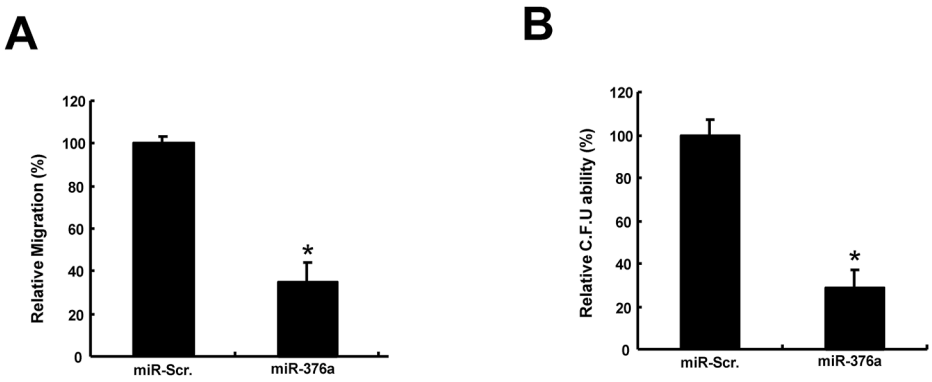

Figure 3 Upregulation of miR-376a has an inhibitory effect on cancer stem cells' phenotypes. Ectopic expression of miR-376a in SAS cancer stem cells mitigated the (A) transwell migration (B) and colony-forming abilities. *$P < 0.05$ compared to miR-Scr.

expression of miR-376a displayed a 70 % reduction in colony-forming ability. Taken together, these findings presented herein attest to the assumption that miR-376a may exert the inhibitory effects on cancer stemness.

Since miRs often regulate target genes by binding to their 3'untranslated regions (3′UTRs) to repress their express, we analyzed the binding sequences of miR-376a and the NRP1 region using bioinformatics databases (miRBase and starBase) and designed luciferase reporter constructs. Fig. 4A displays the complementary base pairing between miR-376a and the 3′ UTR of NRP1. Reporter plasmids, including both wild-type (wt-NRP1) and mutated (mut-NRP1) versions, were generated and subsequently co-transfected into SAS-CSCs with miR-376a mimics. The luciferase reporter assay showed that the relative luciferase activity of wt-NRP1 was significantly reduced after co-transfection of miR-376a mimic, while the mut-NRP1 remained unchanged (Fig. 4B). Moreover, we observed that overexpression of miR-376a resulted in the reduction of NRP1 expression in SAS-CSCs (Fig. 4C). These finding indicated that NRP1 is a potential target of miR-376a.

Subsequently, we investigated whether NRP1 was implicated in the miR-376a-mediated regulation of cancer stemess. Our data showed that overexpression of NRP1 reversed the inhibitory effect of miR-376a mimics on migration ability (Fig. 5A). Another essential feature of CSCs is the ability to divide and generate new CSCs, maintaining the cellular composition of the tumor over time. Hence, we analyzed the self-renewal capacity and found that the overexpression of NRP1 counteracted the suppressive property of miR-376a mimics on self-renewal capacity (Fig. 5B). In summary, these results suggest that miR-376a acts as a tumor suppressor in SAS-CSCs by targeting NRP1.

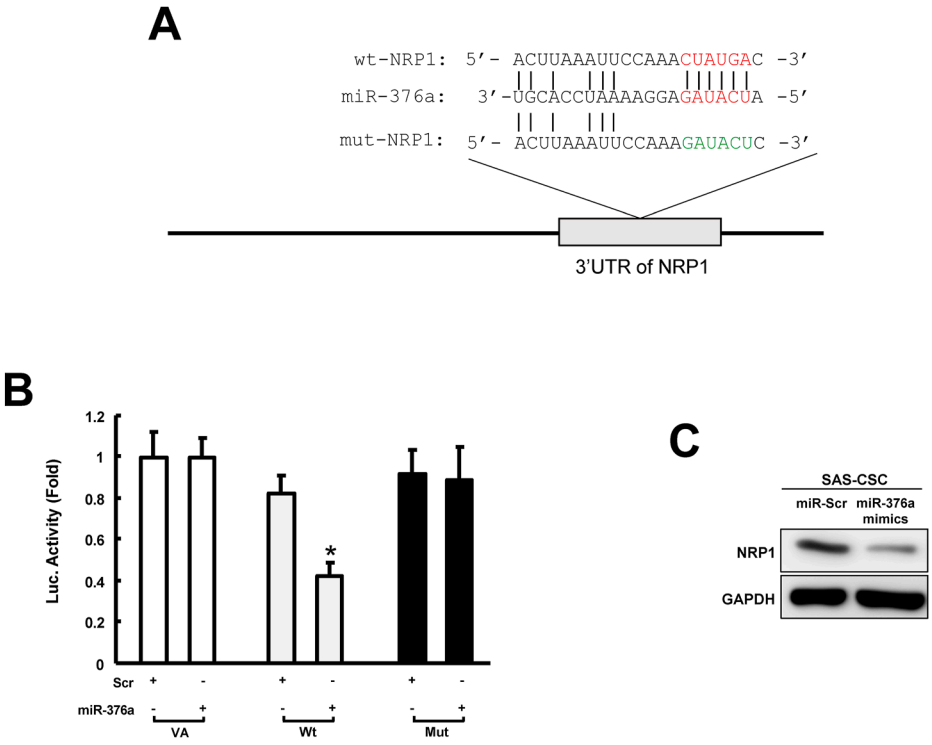

Figure 4 NRP1 is a direct target of miR-376a. (A) The sequences of wild-type neuropilin-1 (wt-NRP1) and mutant NRP1 (mut-NRP1) 3′UTRs along with the miR-376a binding site were presented. (B) MiR-376a mimics decreased the luciferase activity of wt-NRP1 but not that of mut-NRP1. (C) The expression of NRP1 in SAS cancer stem cells transfected with miR-Scr. or miR-376a mimics. *$P < 0.05$ compared to miR-Scr. VA: vector alone; Luc. Luciderase.

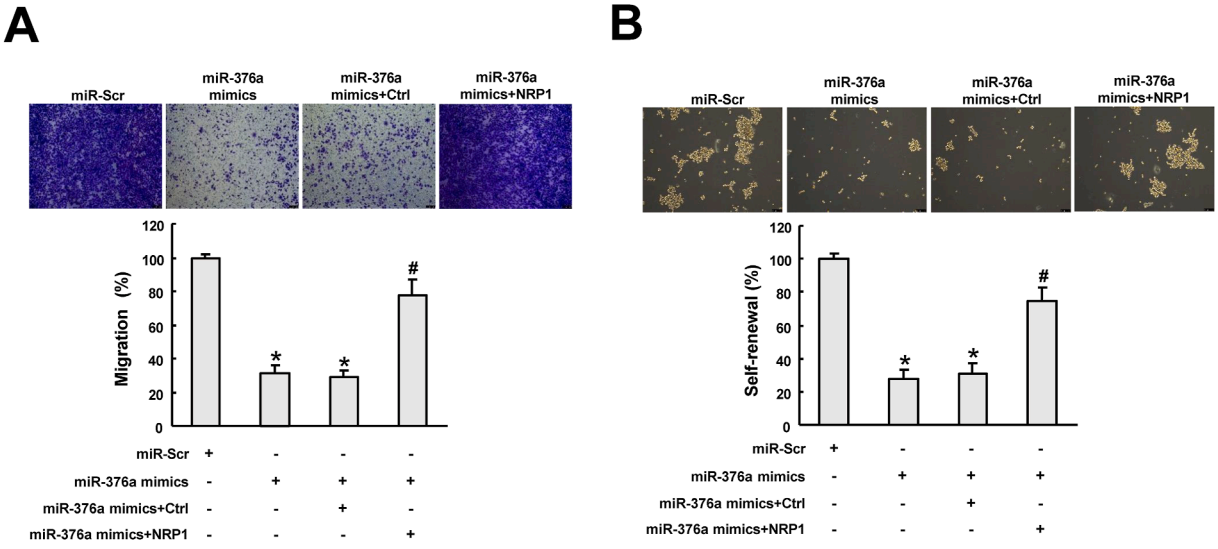

Figure 5 NRP1 overexpression reverses the suppressive effects of miR-376a mimics on cell migration and self-renewal. (A) Transwell migration assay was used to analyze the effects of miR-376a mimics and NRP1 overexpression on cell migration. (B) The self-renewal ability was determined via a secondary sphere formation assay. *$P < 0.05$ compared to mir-Scr group; #$P < 0.05$ compared to miR-376a mimics only group. Ctrl.: vector control.

Discussion

miR-376a was first reported in 2005[32] and was later found to undergo RNA editing that converts adenosine to inosine.[33] It has been shown that reduced adenosine-to-inosine editing of miR-376a* could enhance the invasiveness of glioblastoma cells.[18] In fact, its first identification in cancer was in pancreatic cancer.[34] Previous studies have found that miR-376a plays different roles in various types of cancer. For instance, miR-376a was upregulated in lung cancer[35] and

ovarian cancer,[36] and its upregulation has been shown to drive aggressive lung adenocarcinoma.[21] However, most studies have indicated that miR-376a often functions as a tumor suppressor in cancers such as metastatic prostate cancer,[22] melanoma,[19] and hepatocellular carcinoma.[20] Although studies on the expression and the role of miR-376a in OSCC are still limited, our results suggest that miR-376a likely functions as a tumor suppressor in this context, as its overexpression suppressed the expression of cancer stemness markers and CSC features. As mentioned above, IGF1R[19] and YAP1[24] are the direct targets of miR-376a implicated in cancer stemness. In the present study, we showed that miR-376a may inhibit OSCC stemness by targeting NRP-1.

NRP1 (CD304 or BDCA-4) is a transmembrane glycoprotein that plays a role in a wide range of cellular events, including cell migration, angiogenesis, and axon growth.[37,38] NRP1 is known to interact with various extracellular ligands, such as vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) to promote tumorigenesis.[39] It has been shown that patient-derived glioblastoma multiforme (GBM) cells expressing shRNAs targeting VEGF or NRP1 exhibit lower expression of CSC markers, along with impaired neurosphere formation and migratory ability of CSCs.[40] In GBM cells, autocrine VEGF-VEGFR2 signaling is enhanced through the interaction with NRP1 and the surface VEGFR2 has been shown to promote the self-renewal capacity and viability of CSCs.[41] Another study also revealed that VEGF-A interacted with NRP1 to initiate intracellular signaling events that promote the survival of epidermal CSCs derived from squamous cell carcinoma and drive the formation of aggressive, invasive, and highly vascularized tumors.[42] In triple-negative breast cancer cells, VEGFA is highly expressed in tumor-associated macrophages and tumor cells, and it potentiates the CSC phenotype through NRP1.[43] NRP1 also enhances cancer stemness and promotes radioresistance by facilitating WTAP-mediated m^6A methylation of Bcl-2 mRNA in breast cancer.[44] In addition, the VEGF/NRP1 axis was found to enhance breast cancer progression by inducing epithelial—mesenchymal transition (EMT) and activating the NF-κB and β-catenin signaling pathways.[45] Similar findings were observed in gastric cancer, where the increased NRP1 mRNA stability activated the downstream Wnt/β-catenin signaling pathway and promoted cancer stemness.[46] In OSCC, NRP1 has been found to promote EMT and contribute to the acquisition of CSC-like characteristics through NF-κB activation.[47] Additionally, an interaction between NRP1 and CKLF-like MARVEL transmembrane domain-containing member 6 (CMTM6) has been reported, with NRP1 playing a role in the degradation of CMTM6.[48] Notably, CMTM6 has been revealed to regulate cancer stemness, EMT, and antitumor immunity in head and neck cancer.[49] Future research will further investigate the molecular mechanisms by which NRP1 influences cancer stemness in OSCC.

Within the limitation of this in vitro study only using a single oral cancer cell line (SAS), our results suggest that miR-376a may function as a tumor suppressor in OSCC by regulating NRP1 and thereby influencing cancer stemness. Developing therapeutic strategies targeting the miR-376a/NRP1 axis may help slow the progression of OSCC. Further in vivo studies are warranted to validate these findings and explore clinical implications.

Declaration of competing interest

All authors have no conflicts of interest relevant to this article.

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