miR-532-3p inhibits the progression of tongue squamous cell carcinoma by targeting podoplanin

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Abstract

Background: The association between miR-532-3p and tongue squamous cell carcinoma (TSCC) has been examined in the literature to improve the survival rate of patients with this tumor. However, further studies are needed to confirm the regulatory roles of this microRNA (miRNA) in TSCC. The objective of this study was to investigate the roles played by and the underlying mechanism used by the miR-532-3p/podoplanin (PDPN) axis in TSCC development.

Methods: Western blotting and quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) were performed to evaluate the PDPN expression level in TSCC tissues and cells. The proliferative, adhesive, and migratory capabilities of TSCC cells (CAL-27 and CTSC-3) were examined using cell counting kit-8 (CCK-8), cell adhesion, and wound-healing assays, respectively. The dual-luciferase reporter (DLR) assay was later conducted to confirm the relationship between miR-532-3p and PDPN.

Results: The results indicated that PDPN expression was enriched in TSCC tissues and cells, and that the expression of PDPN was associated with some clinicopathological parameters of TSCC, including lymph node metastasis ($P = 0.001$), tumor-node-metastasis (TNM) staging ($P = 0.010$), and grading ($P = 0.010$). Further analysis also showed that PDPN knockdown inhibited the viability, adhesive ability, and migratory capacity of CAL-27 and CTSC-3 cells, effects that could be reversed by the application of a miR-532-3p inhibitor. Additionally, PDPN was found to be a direct target of miR-532-3p.

Conclusions: This research suggested that by targeting PDPN, miR-532-3p could inhibit cell proliferation viability, adhesion, and migration in TSCC. Findings also revealed that the miR-532-3p/PDPN axis might provide more insights into the prognosis and treatment of TSCC.

Keywords: Tongue squamous cell carcinoma; Podoplanin; miR-532-3p

Introduction

Tongue squamous cell carcinoma (TSCC) is one of the tumors originating in the head and neck, and represents a substantial threat to human health owing to its high rate of recurrence and metastasis.\(^1\) TSCC patients are often diagnosed at an advanced stage, thereby making clinical treatments more difficult.\(^4\) Despite the use of treatment methods such as radiotherapy and chemoradiotherapy, the survival rate of patients with this tumor remains unsatisfactory.\(^5\) Surgery has also proven not to be the best treatment option as it often results in unfavorable clinical outcomes and diminishes the quality of life of TSCC patients.\(^6,7\) For these reasons, urgent research is needed to understand the molecular mechanism underlying the occurrence and development of TSCC.

The podoplanin (PDPN) gene plays an active role in the progression of many cancers. It is a protein-coding gene with high and specific expression in lymphatic endothelial cells, rendering it an effective lymphatic endothelium marker.\(^8,9\) PDPN expression has been observed in various human cancers such as gastric carcinoma\(^10\) and squamous non-small cell lung carcinoma.\(^11\) In gastric carcinoma, high PDPN gene expression indicated poor overall survival and post-progression survival\(^15\) while in squamous non-small cell lung carcinoma, high levels of PDPN expression have been linked to poor clinicopathological features and prognosis.\(^11\) In addition, PDPN has been demonstrated to influence the development of oral cancer.\(^12-17\) However, little or no research has been performed to understand the impact of this gene on TSCC development.

Numerous microRNAs (miRNAs) are known to also influence the development of tumors. They are small, non-coding RNAs with roles in cellular metabolism, inflammation, and carcinomatosis.\(^18-20\) Several abnormally expressed miRNAs have been identified in TSCC. For...
example, miR-488,[21] miR-409-3p,[22] and miR-23b[23] were reported to be significantly downregulated in TSCC, whereas miR-184[24] and miR-611[25] were reported to be upregulated. In one study, miR-532-3p was found to suppress TSCC progression by targeting CC-chemokine receptor 7 (CCR7).[26] However, no study has investigated the effect of miR-532-3p and PDPN on TSCC development.

Here, we investigated the role of PDPN and its relationship with miR-532-3p in the regulation of TSCC progression. The results of this research provide further insights into the multifaceted role of this miRNA in TSCC and reveal that the miR-532-3p/PDPN axis may be a novel therapeutic target for the treatment of TSCC.

Methods

Bioinformatics analysis

An mRNA expression profile GSE34105 from GEO DataSets (https://www.ncbi.nlm.nih.gov/gds/?term=) was used to screen the upregulated genes with log2FC (log2-fold change) >2 and adjusted \( P < 0.05 \). Then, the screened genes were uploaded to STRING (https://string-db.org/) for gene ontology (GO) analysis. To identify the upstream of PDPN, TargetScan (http://www.targetscan.org/vert_71/) and starBase (http://www.sysu.edu.cn) were used to predict the miRNAs binding to PDPN.

TSCC tissue samples

The tumor and adjacent normal tissues used in this study were collected from 27 patients with TSCC at the Sixth Hospital of Wuhan between September 2017 and June 2019. The tissue samples were frozen in liquid nitrogen immediately after they were collected from patients. Informed consent was obtained from the patients before the study, and this research was approved by the Ethics Committee of the Sixth Hospital of Wuhan (No. WHSHIRB-K-2019004). The clinical-stage was classified based on the 8th edition of the American Joint Committee on Cancer Tumor-Node-Metastasis (TNM) classification system.[27] The association of gene expression with the clinicopathological features of TSCC cases are listed in Supplementary Table 1, http://links.lww.com/CM9/A933.

Cell culture and transfection

The human normal oral epithelial cell (HOEC) line was purchased from BeNa Culture Collection (BNCC, Beijing, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (HyClone, USA). The human TSCC cell lines CAL-27 and SCC-25 cell lines, both of which were purchased from Procell Life Science & Technology Co., Ltd (Wuhan, China), were kept in Gibco-provided DMEM and Gibco-provided DMEM/F12, respectively. Another TSCC cell line (CTSC-3) was obtained from ChemicalBook (Shanghai, China) and cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco). All the cell lines were cultured in their respective medium at 37°C in an atmosphere containing 5% CO2. Cell transfection was conducted when the cell grown to 50% confluence using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific, San Jose, CA, USA). Small interfering RNA targeting PDPN (si-PDPN), the miR-532-3p mimic, the miR-532-3p inhibitor, and the negative control (NC) were purchased from RiboBio (Guangzhou, China), and were transfected at a concentration of 30 nmol/L. The transfected cells were collected after 48 h for further analyses.

RT-qPCR assessment

RNA was isolated from CAL-27 and CTSC-3 cells using the mirVana miRNA Isolation Kit (Thermo Fisher Scientific). RNA was then reverse- transcribed into complementary DNA (cDNA) using the one-step miRNA reverse transcription kit (HaiGene, China). qPCR was performed with the GoTaq qPCR Master Mix (Promega, Madison, WI, USA). For PDPN, total RNA was extracted from tissues and cells using the RNAeasy spin column-based Animal RNA Isolation Kit (Beyotime, Guangzhou, China). The extracted RNA was then reverse-transcribed using the BeyoRT II First Strand cDNA Synthesis Kit with gDNA Eraser (Beyotime). qPCR was then performed using the BeyoFast SYBR Green qPCR Mix (2 x, High ROX) (Beyotime) in a Bio-Rad CFX96 Real-Time Detection instrument (Bio-Rad, Hercules, CA, USA). GAPDH and U6 were used as reference genes, and the \( 2^{-\Delta\Delta C_t} \) method was used for quantification. The primer sequences for qPCR are listed in Supplementary Table 2, http://links.lww.com/CM9/A934.

Western blotting analysis

HOEC, CAL-27, CTSC-3, and SCC-25 cells were seeded in 6-well plates (2.5 x 10^5 cells/well) for transfection. After 48 h of transfection, the cells were lysed with pre-cooled radioimmunoprecipitation assay (RIPA) lysis buffer (Pierce, Rockford, IL, USA) containing protease inhibitors. Total protein (40 μg) was separated using 10% SDS-polyacrylamide gel electrophoresis (under 80 V for 20 min and then 100 V for 1 h). Subsequently, the protein was transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% skimmed milk, the membranes were incubated overnight at 4°C with primary antibodies against PDPN (1:1000, Cat#: AV44528, Merck, Rahway, NJ, USA) and β-actin (1:1000, Cat#: A1978, Merck). The next morning, the membranes were incubated with the secondary antibody (1:5000) for 1 h at 25°C. The bands were developed using the enhanced chemiluminescence (ECL) Plus Western Blotting Substrate (Thermo Fisher Scientific) and later analyzed using ImageJ software.

CCK-8 assay

Briefly, approximately 1000 cells/well were seeded into a 96-well plate and transfected when the cell grown to 50% confluence. Then, 10 μL/well of CCK-8 solution (Dojindo, Kumamoto, Japan) was added to the wells and incubated for 60 min. The absorbance of the wells was determined at 450 nm using a microplate reader (Bio Tek, Winooski, VT, USA). The plate was analyzed at intervals of 24 h for 3 days.

Cell adhesion assay

The cell adhesion assay was performed to explore the adhesive ability of CAL-27 and CTSC-3 cells. First, a
96-well plate was prepared by coating it overnight with fibronectin (Sigma-Aldrich, Louis, MO, USA) at 4°C, followed by blocking overnight at 4°C with 1% bovine serum albumin (BSA; Sigma-Aldrich). After that, 5 × 10^4 cells were suspended in serum-free medium, seeded in a 96-well plate, and incubated for 30 or 60 min. Non-adhering cells were washed off with PBS. Next, DMEM containing 10% foetal bovine serum was added to each well, and the mixture was incubated at 37°C for 4 h. A 10 μL aliquot of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) substrate was subsequently added to each well, and the mixture was incubated at 30°C for 2 h. The MTT-treated cells were then dissolved in 100 μL of dimethyl sulfoxide (DMSO). Finally, the absorbance was measured at 570 nm using a microplate reader (Bio Tek).

**Wound-healing assay**

A wound-healing assay was applied to detect the migratory capability of CAL-27 and CTSC-3 cells. Transfected cells (5 × 10^4 cells/well) were first seeded in a 6-well plate and cultured. Next, a scratch was made in the cells using a pipette tip (200 μL), the unattached cells were removed using PBS, and the wound width was observed and photographed under a microscope (Leica, Bensheim, Germany). After 24 h of incubation in serum-free medium, the wound width in each group was again photographed and recorded.

**Dual-luciferase reporter assay**

A dual-luciferase reporter (DLR) assay system (Promega) was employed for multiple comparisons, while a paired two-tailed t test was applied for comparisons between two groups. P values of < 0.05 were considered statistically significant. The association of miR-332-3p and PDPN expression with the clinicopathological features was analyzed by chi-square test or Fisher exact test. All the data were expressed as mean ± standard deviation. Each experiment was performed three times to minimize errors.

**Results**

**PDPN is highly expressed in TSCC tissues and cells**

The GEO dataset GSE34105 was used as the mRNA expression profile for identifying upregulated genes. Using a log2-fold change (FC) > 2 and an adjusted P < 0.05 as a cut-off, a total of 414 upregulated genes were identified and then uploaded to the STRING database (https://string-db.org/) for further gene ontology (GO) analysis. As shown in Figure 1A, we identified five genes (CCL13, CCL19, CCL5, PDPN, and PTPRO) that were associated with cell adhesion, cell proliferation, and cell migration. After reviewing the literature, PDPN was selected as the gene of interest because of its high expression and reported promotive functions in oral cancer.[15,16] RT-qPCR and Western blotting were used to characterize the expression of PDPN in TSCC. As illustrated in Figure 1B, PDPN expression was higher in TSCC tissues (3.95 ± 1.94) than that in non-TSCC tissues (1.00 ± 0.34; P < 0.001). We subsequently divided the clinical samples into low and high PDPN expression groups based on the mean of the PDPN expression in TSCC tissues and then analyzed the correlation between PDPN expression and the clinicopathological features of TSCC. As shown in Supplementary Table 1, http://links.lww.com/CM9/A933, the expression of PDPN was associated with several clinicopathological features of TCC, including lymph node metastasis (P = 0.001), TNM staging (P = 0.010), and grading (P = 0.010). However, the expression of PDPN was not significantly associated with gender (P = 0.343), age (P = 0.883), differentiation (P = 0.153), or tumor localization (P = 0.354). Compared with that in HOEC cells (1.00 ± 0.07), PDPN mRNA levels were significantly increased in CAL-27 (4.04 ± 0.08; P < 0.001), CTSC-3 (4.47 ± 0.14; P < 0.001), and SCC-25 cells (3.70 ± 0.12; P < 0.001) [Figure 1C]. Next, we assessed the change in the PDPN protein expression level, and found that it was increased in CAL-27 (1.86 ± 0.03; P < 0.001), CTSC-3 (1.99 ± 0.05; P < 0.001), and SCC-25 cells (1.61 ± 0.03; P < 0.001) compared with that in HOEC cells (1.000 ± 0.010) [Figure 1D].

**PDPN was downregulated by si-PDPN**

Given that PDPN might be involved in the regulation of TSCC, we used siRNA to knock down PDPN expression in CAL-27 and CTSC-3 cells. The RT-qPCR results showed that, following the transfection of si-PDPN, PDPN expression was decreased by about 70% in CAL-27 cells (0.22 ± 0.02 vs. 1.00 ± 0.05; P < 0.001) and by about 60% in CTSC-3 cells (0.31 ± 0.01 vs. 1.01 ± 0.06; P < 0.001) when compared with that in cells transfected with the blank control [Figure 2A]. Similarly, compared with the blank control group, the protein expression of PDPN was also reduced in si-PDPN-transfected CAL-27 cells (0.64 ± 0.04 vs. 1.00 ± 0.02; P < 0.001) and si-PDPN-transfected CTSC-3 cells (0.74 ± 0.06 vs. 1.00 ± 0.02; P < 0.001) [Figure 2B]. Overall, these results demonstrated that PDPN was downregulated by si-PDPN.

**PDPN knockdown suppressed TSCC development**

Next, we observed the effect of PDPN on TSCC development by assessing the proliferative, adhesive, and migratory capability of TSCC cells transfected with si-PDPN. The results of the CCK-8 assay showed that after a transfection period of 72 h, PDPN knockdown impaired the viability of CAL-27 (0.85 ± 0.05; P < 0.001) and
CTSC-3 cells (1.02 ± 0.07; P < 0.001) compared with that in the blank control group (2.07 ± 0.05 and 2.13 ± 0.04, respectively) [Figure 3A]. A cell adhesion assay demonstrated that the adhesive capacity of si-PDPN-transfected CAL-27 cells was decreased at 60 min compared with that in cells transfected with the blank control (60.60 ± 2.79% vs. 100.00 ± 3.97%; P < 0.001), and a similar result was observed for CTSC-3 cells (70.10 ± 4.54% vs. 100.00 ± 2.52%; P < 0.001) [Figure 3B]. The wound-healing assay results showed that, compared with the blank control group, si-PDPN reduced the migratory ability of CAL-27 and CTSC-3 cells by about 40% (respectively 46.50 ± 1.18% vs. 77.70 ± 4.24%; P < 0.001 and 39.50 ± 2.14% vs. 72.20 ± 3.75%; P < 0.001) [Figure 3C]. Taken together, the results revealed that the knockdown of PDPN by si-PDPN could suppress TSCC development.

**miR-532-3p targeted PDPN in TSCC cells**

TargetScan and starBase were applied to identify which miRNAs bind to PDPN. The results showed 10 overlapping miRNAs between TargetScan and starBase

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**Figure 1:** PDPN expression was increased in TSCC tissues and cells. (A) CXCL13, CCL19, CCL5, PTPRC, and PDPN were screened out, and are associated with cell adhesion, cell proliferation, and cell migration. (B) The mRNA expression of PDPN in TSCC tissues and corresponding non-TSCC tissues as determined by RT-qPCR. (C) The mRNA expression of PDPN in the HOEC and TSCC cell lines (CAL-27, CTSC-3, and SCC-25) as determined by Western blotting. *P < 0.001 vs. HOEC cells. HOEC: Human normal oral epithelial cell; PDPN: Podoplanin; RT-qPCR: Quantitative real-time reverse transcription-polymerase chain reaction; TSCC: Tongue squamous cell carcinoma.
Among them, miR-532-3p attracted our attention because it had the highest score in TargetScan. The binding sites for miR-532-3p on the PDPN 3'UTR are shown in Figure 4B. The predicted relationship between miR-532-3p and PDPN was further determined through a luciferase reporter assay. As depicted in Figure 4C, luciferase activity decreased in CAL-27 and CTSC-3 cells co-transfected with the miR-532-3p mimic and WT of PDPN (PDPN-WT) (0.37 ± 0.05; *P < 0.001 and 0.45 ± 0.02; *P < 0.001, respectively) compared with that in CAL-27 and CTSC-3 cells co-transfected with the NC and PDPN-WT (1.00 ± 0.02 and 1.00 ± 0.02, respectively). This indicated that miR-532-3p could bind to the PDPN 3'UTR.

Next, we characterized the changes in miR-532-3p expression in TSCC progression. As illustrated in Figure 4D, miR-532-3p expression was significantly reduced in TSCC tissues when compared with that in non-TSCC tissues (0.36 ± 0.13 vs. 1.00 ± 0.43; *P < 0.001). We further found that there was a negative correlation between miR-532-3p and PDPN in TSCC tissue (R² = 0.66; *P < 0.001) [Figure 4E]. Subsequently, we divided the clinical samples into low and high miR-532-3p expression groups based on the mean miR-532-3p expression in TSCC tissues and assessed whether there was a correlation between miR-532-3p expression and the clinicopathological features of TSCC. The result showed that the expression of miR-532-3p was significantly associated with lymph node metastasis (*P = 0.025), TNM staging (*P = 0.004), and grading (*P = 0.001), but not with gender (*P = 0.863), age (*P = 0.148), differentiation (*P = 0.405), or tumor localization (*P = 0.441) [Supplementary Table 1, http://links.lww.com/CM9/A933]. Besides, compared with HOEC cells (1.00 ± 0.12), the levels of miR-532-3p were significantly reduced in CAL-27 (0.28 ± 0.03; *P < 0.001), CTSC-3 (0.17 ± 0.02; *P < 0.001), and SCC-25 cells (0.35 ± 0.04; *P < 0.001) [Figure 4F]. The transfection efficiency of miR-532-3p is depicted in Figure 4G. Compared with the CON group (1.00 ± 0.05 in CAL-27 cells and 1.00 ± 0.04 in CTSC-3 cells), the expression level of miR-532-3p was decreased in the inhibitor-treated cells (0.29 ± 0.03; *P < 0.001 in CAL-27 cells and 0.38 ± 0.01; *P < 0.001 in CTSC-3 cells), but increased in those treated with the miR-532-3p mimic (3.41 ± 0.30; *P < 0.001 in CAL-27 cells, and 3.03 ± 0.35; *P < 0.001 in CTSC-3 cells). PDPN protein expression was also detected using Western blotting. The result revealed that PDPN expression was significantly enhanced by the miR-532-3p inhibitor in CAL-27 and CTSC-3 cells (1.86 ± 0.07; *P < 0.001 and 1.77 ± 0.06; *P < 0.001, respectively) compared with that in the blank control group (1.00 ± 0.02 and 1.00 ± 0.06, respectively), while it was reduced by the miR-532-3p mimic in CAL-27 and CTSC-3 cells (0.21 ± 0.03; *P < 0.001 and 0.76 ± 0.02; *P < 0.001, respectively) [Figure 4H].

**miR-532-3p inhibitor treatment reversed the tumor inhibitory effect of PDPN knockdown**

Rescue experiments were designed to understand the interaction between miR-532-3p and PDPN in TSCC cells. CCK-8, cell adhesion, and wound-healing assays were
carried out to examine CAL-27 and CTSC-3 cells treated with si-PDPN or si-PDPN plus the miR-532-3p inhibitor. The CCK-8 assay result indicated that cell viability was increased in the si-PDPN + miR-532-3p inhibitor group (1.78 ± 0.07; P < 0.001 in CAL-27 cells and 1.91 ± 0.08; P < 0.001 in CTSC-3 cells) compared with that in the si-PDPN only group (1.02 ± 0.07 in CAL-27 cells and 1.13 ± 0.06 in CTSC-3 cells) after 72 h of transfection [Figure 5A]. The results of the cell adhesion assay showed that when si-PDPN was co-transfected with the miR-532-3p inhibitor, the inhibitory effect of si-PDPN on cell adhesion could be reversed (81.30 ± 2.21% vs. 61.36 ± 3.33%; P < 0.001 in CAL-27 cells and 88.10 ± 3.80% vs. 72.23 ± 1.78%; P < 0.001 in CTSC-3 cells) [Figure 5B]. Similarly, the wound-healing assay result demonstrated that in CAL-27 and CTSC-3 cells, the level of cell migration in the si-PDPN + miR-532-3p inhibitor group (70.60 ± 2.13%; P < 0.001 and 61.30 ± 4.29%;
Figure 4: PDPN was identified as a target gene of miR-532-3p in TSCC cells. (A): Ten miRNAs targeting PDPN overlapped between TargetScan and starBase. (B): The predicted binding sites for miR-532-3p on the PDPN 3′ UTR. (C): A DLR assay was performed to verify the binding of miR-532-3p to PDPN. WT: Wild-type; MUT: Mutant; NC: miR-532-3p mimic NC. \( P < 0.001 \) vs. the miR-532-3p mimic NC. (D): The relative expression of miR-532-3p in TSCC tissues and corresponding non-TSCC tissues as determined by RT-qPCR. (E): The correlation between miR-532-3p and PDPN in TSCC tissues was analyzed by Pearson’s correlation. (F): The relative expression of miR-532-3p in the HOEC and TSCC cell lines (CAL-27, CTSC-3, and SCC-25) as determined by RT-qPCR. (G): The transfection efficiency of the miR-532-3p inhibitor and mimic in CAL-27 and CTSC-3 cells. \( P < 0.001 \) vs. the blank control group. (H): The protein expression of PDPN in CAL-27 and CTSC-3 cells after the transfection of the miR-532-3p inhibitor and mimic. CON: Blank control group; DLR: Dual-luciferase reporter; HOEC: Human normal oral epithelial cell; miRNAs: MicroRNAs; NC: Negative control; PDPN: Podoplanin; RT-qPCR: Quantitative real-time reverse transcription-polymerase chain reaction; TSCC: Tongue squamous cell carcinoma. \( P < 0.001 \) vs. the blank control group.
Figure 5: The miR-532-3p inhibitor could reverse the tumor inhibitory effect of PDPN knockdown. The cells were transfected with si-PDPN or si-PDPN plus the miR-532-3p inhibitor. (A) A CCK-8 assay was performed to detect the viability of CAL-27 and CTSC-3 cells. (B): A cell adhesion assay was performed to assess the adhesive ability of CAL-27 and CTSC-3 cells. (C) A wound-healing assay was performed to evaluate the migratory ability of CAL-27 and CTSC-3 cells. CCK-8: Cell counting kit-8; CON: Blank control group; NC: Negative control; PDPN: Podoplanin. *P < 0.05 vs. the blank control group.
Although we demonstrated the impact of the miR-532-3p/PLEDIN axis on TSCC development, this study had several limitations. The effect of the miR-532-3p/PLEDIN axis on TSCC development involves a complex regulatory network, including signaling pathways. However, we did not explore this complexity, and future research should examine this aspect. Also, in vivo experiments were not conducted in this study due to limited funds. These limitations should be considered in future studies on TSCC.

Collectively, our results demonstrated that miR-532-3p plays a crucial role in TSCC progression by targeting PLEDIN. Furthermore, PLEDIN was observed to be significantly enriched in TSCC tissues, and could enhance the adhesive capacity, viability, and migratory ability of TSCC cells. However, the promotive effect of PLEDIN on TSCC cells could be relieved by miR-532-3p. These findings suggested that the miR-532-3p/PLEDIN axis might be a novel therapeutic target for the treatment of TSCC.

Conflicts of interest

None.

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