miR-139-5p suppresses the proliferation of oral squamous cell carcinoma cells and targeting TPD52

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Abstract: MicroRNA-139-5p (miR-139-5p) has been reported to be frequently downregulated in various types of human cancer. However, the precise molecular mechanism of miR-139-5p underlying oral squamous cell carcinoma (OSCC) remains largely unknown. Here, the results showed that, miR-139-5p was significantly downregulated in OSCC tumor tissues and cell lines. Overexpression of miR-139-5p inhibited cell proliferation of OSCC cells whereas the downregulation of miR-139-5p promoted cell proliferation. A direct interaction between miR-139-5p and 3'-UTR of TPD52 was validated by dual-luciferase reporter assay. Moreover, overexpression of miR-139-5p in OSCC cells significantly decreased the expression of TPD52 and downregulation of miR-139-5p inhibited the expression of TPD52. In addition, the miR-139-5p over expressing OSCC cells exhibited a low growth rate in the xenograft tumor assay. Therefore, the results suggest that miR-139-5p is a tumor suppressor in OSCC through targeting TPD52, which serves as a promising therapeutic target for the treatment of OSCC.

Keywords: MiR-139-5p, TPD52, oral squamous cell carcinoma

Introduction

Oral squamous cell carcinoma (OSCC) is a kind of oral cancer, which causes approximately 500,000 deaths annually [1]. The current treatment for OSCC, including surgery, radiation and chemotherapy. Recently, the molecular targeted therapies are becoming as a novel approaches with potential advantages for the treatment of various cancers, including OSCC [2]. Therefore, it is worthwhile to elucidate the detailed molecular mechanisms.

miRNAs play crucial roles in various biological processes and dysfunction of miRNAs has been shown to be involved in tumorigenesis, including OSCC [3, 4]. For example, it has reported that miR-21, miR-125b-2*, miR-134, miR-155, miR-184 and miR-205 are associated with OSCC clinical pathology [5]. The proliferation and invasion of OSCC cells were increased when miR-329 and miR-410 was downregulated [6]. And overexpression of miR-125a can inhibit the proliferation and invasion of OSCC cells through targeting estrogen-related receptor α [7]. miR-139 is located on chromosome 11q13.4 within the second intron of the phosphodiesterase 2A (PDE2A) gene. MiR-139-5p is a common type of mature miRNA generated from a miR-139 pre-cursor [8]. However, the role of miR-139-5p in OSCC cells remains unclear.

Tumor protein D52 (TPD52) is the founding member of the TPD52 protein family, which was originally identified increased in human breast carcinoma and further found overexpression in a variety of malignancies, such as medulloblastoma, lung and prostate cancer patients [12, 8]. Overexpression of TPD52 has been demonstrated correlated with poor prognosis in breast and prostate cancer patients [9]. Moreover, TPD52 overexpression can serve as a favorable prognostic marker for ovarian carcinoma patients [10]. Cell line and in vivo experiments showed that TPD52 can promote tumorigenesis and metastasis [13].

Here, we investigated the role of miR-139-5p in several OSCC-derived cell lines using exogenous over-expression and knock-down approa-
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Our results indicate that miR-139-5p was downregulated in OSCC tissues and cell lines. MiR-139-5p also suppressed OSCC cell proliferation in vitro and in vivo. Furthermore, we identified that TPD52 was a direct target of miR-139-5p. The results we identified here suggest that miR-139-5p is a tumor suppressor through targeting TPD52, which may serve as a promising therapeutic target for the treatment of OSCC.

Methods and materials

Patients and specimens

A total of 8 paired OSCC and adjacent nontumor specimens were collected from the central hospital of Wuhan. All tissue samples were freshly frozen in liquid nitrogen immediately after collection and stored at -80°C until use. The study protocol was approved by Ethical Committee of the central hospital of Wuhan. Informed consent was obtained from all patients. Both tumor and non-tumor samples were confirmed by pathological examination.

Cell culture and animals

OSCC cell lines, including SCC-4, HSC3, OEC-M1, KB, OC3 (human oral squamous cell carcinoma-derived cell lines) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured according to the manufacturer’s instructions. Normal human oral keratinocytes (HOK) were obtained from ScienCell (Carlsbad, CA, USA) and cultured in oral keratinocyte medium according to the manufacturer’s instructions. The cells were maintained in a humidified atmosphere containing 5% CO$_2$ at 37°C.

Male BALB/c nude mice (weighed 25-30 g) which is five-week-old were purchased from the Laboratory Animal Centre of the central hospital of Wuhan and were raised and handled according to the guideline of the Institutional Animal Care and Use Committee of the central hospital of Wuhan.

MiRNA mimics and cell transfection

The miR-139-5p mimics (miR-139-5p) and negative control miRNA mimics (miRNANC), Anti-NC, Anti-miR-139-5p (Anti-139-5p) were synthesized by GenePharma (China). Transfection was carried out using Lipofectamine 2000 (Invitrogen, UK) method. The final concentration was 100 nM for mimics.

Cell proliferation assay

Cell viability was detected by MTT assay. Briefly, cells were seeded into a 96-well plate at 1×10$^3$ per well for 0, 24, 48, 96, 192 h. The MTT was added according to the instructions and the cells were continually cultured for another 4 h, the medium was discarded and dimethylsulfoxide (150 µl/well; Sigma, St. Louis, MO, USA) was added to dissolve the formed formazan crystals. The optical density at 490 nm was then measured with a microtiter plate reader (Thermo Electron Corporation, vantaa, Finland).

Luciferase reporter assay

The 3'UTR of TPD52 in the pMir-GLO reporter vector was purchased from Clontech, Mountain View, CA, USA. The miR-139-5p binding site was mutated using Quik Change Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). WT (GALNT4 3'UTR wt) and mutant constructs (GALNT4 3'UTR mut) were transfected into OC3 and OEC-M1 cells concurrently with miR-139-5p mimics or mimics control. At 48 hours after transfection, cells were assayed for relative luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Transfections were conducted in triplicate.

OC3 cells were seeded into 24-well plates at 2×10$^5$ per well. TPD52-3'UTR or TPD52-3'UTR mut (100 ng) was co-transfected with 100 nM miR-139-5p or miR-NC control using Lipofectamine 2000. Renilla and firefly luciferase activities were measured by Dual-Luciferase Reporter Assay (Promega) at 36 h after transfection.

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) and cDNA was generated using M-MLV reverse transcriptase (Clontech, Palo Alto, CA, USA) and One Step Prime Script miRNA cDNA Synthesis kit (Takara, Dalian, China), respectively, according to the manufacturer’s instructions. The gene expression was detected using SYBR-Green qPCR Master Mix (Thermo Fisher, Shanghai,
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China). The relative quantification of the gene expression level was compared with the internal reference U6 SnRNA using the $2^{-\Delta\Delta Ct}$ method.

Figure 1. MiR-139-5p was reduced in OSCC. A. RT-qPCR analysis of miR-139-5p expression between OSCC and matched adjacent non-tumorous tissues from OSCC patients; B. RT-qPCR analysis of miR-139-5p in different cell lines, including SCC-4, HSC3, OEC-M1, KB and OC3 cell lines. HOK cells were used as control. OSCC, oral squamous cell carcinoma; HOK, human oral keratinocytes.

Figure 2. MiR-139-5p inhibits the cell viability of OSCC cells. RT-qPCR analysis of miR-139-5p expression in (A) OC3 and (C) OEC-M1 cells infected with miR-139-5p mimics (miR-139-5p) or anti-miR-139-5p (anti-139-5p). The MTT assays were performed with miR-138 overexpression or downregulation at 0, 24, 48, 96 and 192 h in OC3 (B) and OEC-M1 cells (D).
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Statistical analysis

Statistical analyses were performed by one-way ANOVA. P≤0.05 was considered as statistically significant differences (GraphPad Prism 5; GraphPad software, Inc., San Diego, CA, USA).

Results

Expression levels of miR-139-5p in HCC tissues and cell lines

To examine the role of miR-139-5p in OSCC, the expression profile of miR-139-5p in OSCC patient samples and matched non-cancerous epithelia was examined with RT-qPCR analysis. The results showed that miR-139-5p was significantly downregulated in 8 OSCC tumor tissues as compared with that in the adjacent non-tumorous tissues (Figure 1A). In addition, we detected the expression of miR-139-5p in several OSCC cell lines, which showed that miR-139-5p expression was markedly reduced to different degrees in SCC-4, HSC3, OEC-M1, KB and OC3 cell lines than that in the control HOK cells (Figure 1B).

MiR-139-5p suppresses cell viability of OSCC cells

Considering that low levels of miR-139-5p expression is expressed in OSCC tissues, we asked that if miR-139-5p function as tumor suppressor. As shown in Figure 2A and 2C, we used miR-139-5p and anti-139-5p to transfect OC3 and OEC-M1 cells, which showed efficient suppression of OSCC cell growth in vitro and in vivo. Overexpression of miR-139-5p significantly decreased the number of colony in OC3 (A, B) and OEC-M1 (D, E); Overexpression of miR-139-5p significantly decreased tumor weight in OC3 (C) and OEC-M1 (F).
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transfection efficiency. The MTT assay showed that overexpression of miR-139-5p significantly inhibits cell viability in both OSCC cell lines; furthermore, knockdown of miR-139-5p significantly increased the cell viability (Figure 2B and 2D).

MIR-139-5p suppresses OSCC cell growth in vitro and in vivo

Furthermore, to evaluate a longer-time impact, we performed the colony formation assay and in vivo assay. Overexpression of miR-139-5p significantly decreased the clonogenic ability in tumor cells (Figure 3A, 3B, 3D, 3E). To further evaluate the potential effect of miR-139-5p on the proliferation of OSCC cells in vivo, OC3 and OEC-M1 cells were transfected with miR-139-5p mimics and then injected into the dorsal flank of nude mice. The results showed that, as compared to the miR-NC group, the mean tumor weight of the miR-139-5p group was markedly reduced (Figure 3C and 3F). These data suggested that miR-139-5p inhibited OSCC proliferation in vitro and in vivo.

MiR-139-5p directly targets TPD52

Since miRNAs mainly function through inhibiting their target mRNAs by binding to the 3'UTR, we searched the putative target genes of miR-139-5p in online miRNA target prediction databases (Targetscan and microRNA.org), and found that TPD52 is a potential target of miR-139-5p. A dual-luciferase reporter system was used with co-transfection of miR-139-5p and a luciferase reporter plasmid containing a wild-type or mutant 3'UTR of human TPD52 (Figure 4A). Luciferase activity of wild-type TPD52 3'UTR was significantly inhibited by miR-139-5p, while miR-139-5p failed to inhibit the expression of luciferase constructs with mutated target sites, suggesting that miR-139-5p directly targets the 3'UTR of TPD52 (Figure 4B). Moreover, a change in mRNA expression levels of TPD52 in response to miR-139-5p overexpression or inhibition was verified by RT-qPCR in OC3 and OEC-M1 cells (Figure 4C and 4D), confirming that miR-139-5p negatively regu-
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lates TPD52 expression by directly targeting their 3’UTR regions.

Discussion

In the present study, we have reported that miR-139-5p was decreased in OSCC tumor tissues and OSCC cells. Our results confirmed that miR-139-5p suppressed OSCC cell proliferation in vitro and in vivo. Moreover, to the best of our knowledge, for the first time, we have delineated a direct interaction of miR-139-5p and TPD52 that may be involved in the regulation of OSCC.

Previous studies have revealed that miR-139-5p serves as a tumor suppressor in several human cancers. For example, Krishnan et al. found that miR-139-5p could inhibit the invasion and migration of breast cancer cells [15]. And studies revealed that miR-139-5p was down-regulated in esophageal squamous cell carcinoma (ESCC), colorectal cancer (CRC) tissue [16]. Consistent with previous studies, we found that miR-139-5p was under-expressed in all OSCC cell lines and tissue samples. However, the mechanism by which miR-139-5p is down-regulated in cancer tissues is still controversial. Au et al. found that EZH2 regulated H3K27 methylation which repressed miR-139-5p expression in human HCC through [17]. Furthermore, it has been demonstrated that miR-139-5p was down-regulated in colorectal cancer tissue, while the pre-miR-139 expression level was unchanged compared to non-cancerous samples [18], which indicate that there may be a post-transcriptionally regulatory mechanism occurring during miR-139 maturation.

Furthermore, studies have suggested that miR-139-5p can induced cell cycle arrest in the G0/G1 phase and suppressed the invasion of esophageal carcinoma cells through targeting the NR5A23’UTR [19]. Song et al. identified that miR-139-5p can inhibit migration and invasion of colorectal cancer via suppressing the expression of AMFR and NOTCH1 [20]. Here, we demonstrated that miR-139-5p can suppress the cell viability and cell growth with MTT and colony formation assays. And in vivo assays further demonstrated that miR-139-5p can suppress the proliferation the OSCC.

In our study, luciferase reporter assays detected direct binding of miR-139-5p to the 3’UTR of TPD52 transcripts. Furthermore, over-expression of miR-139-5p decreased the level of TPD52. Taken together, it seems that miR-139-5p serves as a pivotal mediator in regulation of OSCC proliferation.

In summary, we have found a down-regulation of miR-139-5p in OSCC tissues and cell lines. MiR-139-5p suppressed OSCC cells proliferation in vitro and in vivo. Furthermore, TPD52 was identified as a direct target of miR-139-5p. Thus, miR-139-5p may be a diagnostic marker and a potential target of anti-cancer therapy of OSCC.

Disclosure of conflict of interest

None.

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