miR-148a modulates the viability, migration and invasion of oral squamous cell carcinoma cells by regulating HLA-G expression

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Abstract. Oral squamous cell carcinoma (OSCC) is a common malignancy of the oral and maxillofacial regions. MicroRNAs (miRs) are a group of endogenous small noncoding RNAs that inhibit gene expression by binding to the mRNA of target genes, and serve important roles in numerous biological processes. Reverse transcription-quantitative polymerase chain reaction and western blotting were used to detect gene and protein expression levels, respectively. Cell proliferation, migration and invasion were detected using MTT, wound healing and Matrigel assays, respectively. The association between miR-148a and human leukocyte antigen-G (HLA-G) was analyzed using Targetscan and Luciferase reporter assays. In the present study, miR-148a was revealed to be significantly downregulated in OSCC cells. To further investigate the functions of miR-148a in OSCC, the viability, migration, and invasive abilities of SCC-9 cells were investigated following transfection with miR-148a mimics or miR-148a inhibitor. It was revealed that transfection with miR-148a mimics significantly reduced the viability, migration and invasion of cells, whereas miR-148a inhibitor significantly enhanced these properties. In addition, HLA-G was identified as a direct target of miR-148a and demonstrated to be downregulated in OSCC cells. Furthermore, it was revealed that transfection with miR-148a mimics decreased the expression levels of HLA-G mRNA and protein in SCC-9 cells, whereas transfection with miR-148a inhibitor increased the expression of HLA-G mRNA and protein. The results indicated that there was an association between miR-148a and HLA-G expression, and suggested that miR-148a may be a potential target in the treatment of OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is a common malignancy of the oral and maxillofacial regions (1-3). Local infiltration and distant metastasis are the leading causes of patient mortality (4). Numerous studies have reported that the development of tumors is dependent upon the interactions between cancer cells and the tumor microenvironment, in addition to the malignant proliferation of cancer cells (5). The microenvironment is associated with the invasion, metastasis and prognosis of OSCC (6). Investigation into the involvement of human leukocyte antigens (HLAs) in tumor development is important for improved understanding of tumor immunology (7). HLA-G is a non-classical HLA Class-Ib molecule and was first identified in placental extravillous trophoblast (EVT) cells (8,9). It has been demonstrated that HLA-G serves an important role in maternal-fetal immune tolerance (10). HLA-G is an immune tolerance molecule that binds to the immunosuppressive receptors of natural killer (NK) and T cells to inhibit these cells, and induce immunosuppressive signaling (11). The study of HLA-G has expanded from reproduction to organ transplantation, autoimmune diseases and tumor research (12-15), with the role of HLA-G in the immune invasion of tumors of particular interest.

MicroRNAs (miRNAs/miRs) are a group of endogenous small noncoding RNAs (21-23 nucleotides in length) in eukaryotes (16). miRNAs do not encode proteins, but inhibit the expression of genes by binding to the 3'-untranslated regions (3'-UTRs) of target mRNAs (17-19). It was reported that the abnormal expression of miR-148a was associated with the occurrence and development of various tumors, such as ovarian and non-small cell lung cancer (20,21). Therefore, the expression of miR-148a is considered to possess potential clinical value.

Previous studies have reported that HLA-G is a target of miR-148a (22-24). HLA-G serves an important role in the development and metastasis to lymph nodes of OSCC (25). Increased expression of HLA-G in metastatic OSCC is associated with poor clinical prognosis (26). These findings indicated that miR-148a may serve important roles in OSCC; however, the expression and effects of miR-148a in OSCC remain unclear. In the present study, the expression of miR-148a in an OSCC cell line and normal oral cell line was determined, and its effects on the viability, migration and invasion of OSCC...
cells was investigated. The results indicated that miR-148a may serve as a novel molecular target in the diagnosis and treatment of OSCC.

Materials and methods

Cell culture and cell transfection. The OSCC cell line SCC9 and the primary normal human oral keratinocyte (HOK) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The human OSCC cell line, SCC-9, was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and Ham's F12 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 1.2 g/l sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES and 0.5 mM sodium pyruvate supplemented with 400 ng/ml hydrocortisone and 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated in a humidified incubator at 37°C with 5% CO₂. SCC-9 cells were transfected with 50 nM miR-148a inhibitor (miR-148a inhibitor, 5'-aca aaG uGa cac Guu cGG aGa aTT-3') and reverse, 5'-AGGTTCUGUACUGCUGAUU-3') or 50 nM negative control miRNA (NC) (NC forward, 5'-UUCUCCAGUGUGUCAGUTT-3' and reverse, 5'-ACG UGACGUCCUGGAATT-3') using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h according to the manufacturer's protocols. miR-148a inhibitor, mimic and the NC were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols and stored at -80°C. The total RNA concentration was determined using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Total RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. qPCR was performed using SYBR Green Premix Ex Taq (Takara Bio, inc., Otsu, Japan) according to the manufacturer’s protocol. qPCR were as following: GAPDH, forward, 5'-TTTGTGATCGTTGAAGGACCT-3' and reverse, 5'-TGAGGCGAGGATGATGTCTT-3'; U6, forward, 5'-GCTTCCGAGGCGAT ATACTAAAT-3' and reverse, 5'-CGGCTACAGATTTTGCGTGC AT-3'; miR-148a, forward, 5'-CTGTACACGAGACTTTGT-3' and reverse, 5'-AGTGTACAGACGCTGT-3'; HLA-G, forward, 5'-GAGGAGACAGGGAACACCAAG-3' and reverse, 5'-GGTTCGACCAATCATTCCACT-3'; β-catenin, forward, 5'-GCTTTCATGTTGACTGACCA-3' and reverse, 5'-CAAGTCCAGATCAGCAGTC-3'; matrix metalloproteinase-7 (MMP7), forward, 5'-TGTGGAAGATGACCTTCTGGCTGT-3' and reverse, 5'-TGTGACAATCGTTTCTGTCGAC-3'; and T-cell factor-4 (TCF-4), forward, 5'-CAG TCTTCCCTCGGTCT-3' and reverse, 5'-CCTCCGGTCTT-3'. The relative expression levels of genes were normalized to U6 for miRNA or GAPDH for mRNA, and calculated using the 2-ΔΔcq method (27). All experiments were performed in triplicate.

Western blot analysis. Cells were washed three times with cold PBS and total cellular proteins were extracted using a modified radioimmunoprecipitation assay buffer (OriGene Technologies, Inc., Beijing, China) with 1 mM phenylmethylsulfonyl fluoride for 30 min. A bicinechoninic acid protein assay kit (Thermo Fisher Scientific, Inc.) was used to measure the protein concentration. Equal amounts of protein (30 µg/lane) were separated via 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk at room temperature for 1.5 h, followed by incubation overnight at 4°C with the following primary antibodies against: HLA-G (1:1,000; ab135736; Abcam, Cambridge, UK), MMP7 (1:1,000; cat. no. 3801; Cell Signaling Technology, Inc., Danvers, MA, USA); β-catenin (1:1,000; cat. no. 8480, Cell Signaling Technology, Inc.); TCF-4 (1:1,000; cat. no. 2565; Cell Signaling Technology, Inc.) and GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.). The membranes were subsequently incubated for 2 h at room temperature with an anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.). Protein bands were visualized using an enhanced chemiluminescence kit (Applygen Technologies, Inc., Beijing, China). GAPDH was used as an internal control.

MTT assay. An MTT assay was performed to determine the viability of cells. Cells were inoculated in a 96-plate with 1x10⁴ cells/well and incubated at 37°C with 5% CO₂ for 12, 24 or 48 h. In detail, 20 µl MTT reagent (5 mg/ml) was added to the wells. Following incubation for a further 4 h at 37°C, the formazan crystals were dissolved in 150 µl dimethyl sulfoxide and agitated for 10 min. The absorbance at 490 nm was detected using a microplate reader.

Matrigel® invasion assay. A Matrigel-based invasion assay was performed to evaluate the invasive ability of cells. The wells on the upper chamber of Transwell plates were coated with Matrigel. DMEM was then added to the upper chamber and incubated at 37°C for 1 h. Cells were seeded in the upper chamber at a density of 5x10⁴ cells/well in 100 µl serum-free DMEM. DMEM medium containing 20% FBS (Gibco; Thermo Fisher Scientific, Inc.) was added to the lower chamber (24-well plate). The cells were incubated at 37°C for 24 h; then, the lower membranes of the chamber were fixed with 4% paraformaldehyde at room temperature for 30 min and stained with 0.1% crystal violet at room temperature for 20 min. Images of five randomly selected fields per view were captured using a light microscope (magnification, x200) and the numbers of cells were counted.

Luciferase reporter assay. TargetScan 7.1 bioinformatics software (www.targetscan.org/vert_71) was used to predict the targets of miR-148a; it was revealed that HLA-G was a potential target of miR-148a. To investigate the association between miR-148a and HLA-G, the wild-type (WT) and mutant (MUT) 3'-UTRs of HLA-G were cloned into pmiR-RB-Report™ dual-luciferase reporter gene plasmid.
OSCC cells following transfection with miR-148a mimics (**P<0.01 vs. nc). (c) relative expression of miR-148a in OSCC cells following transfection with miR-148a as determined by reverse transcription-quantitative polymerase chain reaction analysis (**P<0.01 vs. normal cell). (B) relative expression of miR-148a in OSCC cells following transfection with miR-148a mimics (**P<0.01 vs. NC). Data are presented as the mean ± standard deviation. oScc, oral squamous cell carcinoma; control, non-transfected cells; miR-148a mimics (**P<0.01 vs. nc). 

Figure 1. mir-148a is downregulated in oScc cells. (a) relative expression of mir-148a in cancer cells (Scc-9) and normal cells (human oral keratinocytes), mir-148a, microrna-148a; inhibitor, mir-148a inhibitor; mimics, miR-148a mimics; nc, negative control.

Expression of miR-148a in OSCC. To investigate the role of miR-148a in OSCC, RT-qPCR analysis was performed to determine the relative expression of miR-148a in the human OSCC cell line, SCC-9 and normal human oral keratinocytes (HOK). It was revealed that miR-148a was significantly downregulated in SCC-9 cells compared with HOK cells (Fig. 1A). To investigate the effects of miR-148a on SCC-9 cells, cells were transfected with NC, miR-148a mimics or miR-148a inhibitor for 48 h. RT-qPCR analysis was conducted to determine the expression levels of miR-148a. It was demonstrated that miR-148a mimics significantly upregulated the expression of miR-148a in SCC-9 cells compared with the control, whereas miR-148a inhibitor exhibited opposing effects (Fig. 1B and C).

Effects of miR-148a on the viability, migration and invasion of OSCC cells. The viability of SCC-9 cells following transfection was analyzed using an MTT assay. It was demonstrated that miR-148a mimics significantly reduced the viability of cells compared with the control (Fig. 2A). The migration of cells was determined via a scratch-wound assay. Cell migration was markedly reduced following transfection with miR-148a mimics compared with the control (Fig. 2B). Additionally, a Matrigel invasion assay demonstrated that the invasive ability of SCC-9 cells was notably decreased following the overexpression of miR-148a (Fig. 2C). Conversely, transfection with miR-148a inhibitor induced opposing effects on the viability, migration and invasion of SCC-9 cells.

Effects of miR-148a on the expression of invasion and migration-associated proteins in oral squamous cell carcinoma. To further investigate the effects of miR-148a on the migration and invasion of SCC-9 cells, the expression of proteins involved in the Wnt/β-catenin signaling pathway was analyzed. It was revealed that overexpression of miR-148a markedly downregulated the expression of HLA-G, β-catenin, MMP-7 and TCF-4 at the protein level, but significantly downregulated their expression at the mRNA level (Fig. 3A-J).

HLA-G is direct target gene of miR-148a. To further investigate the effects of miR-148a on the migration and invasion of SCC-9 cells, the expression of proteins involved in the Wnt/β-catenin signaling pathway was analyzed. It was revealed that overexpression of miR-148a markedly downregulated the expression of HLA-G, β-catenin, MMP-7 and TCF-4 at the protein level, but significantly downregulated their expression at the mRNA level (Fig. 3A-J).

Statistical analysis. Data were presented as the mean ± standard deviation of at least three independent experiments. Differences between groups were analyzed using Student’s t-tests or one-way factorial analyses of variance followed by a Tukey’s post-hoc test. P<0.05 was considered to indicate a statistically significant difference. Data were analyzed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Expression of miR-148a in OSCC. To investigate the role of miR-148a in OSCC, RT-qPCR analysis was performed to determine the relative expression of miR-148a in the human OSCC cell line, SCC-9 and normal human oral keratinocytes (HOK). It was revealed that miR-148a was significantly downregulated in SCC-9 cells compared with HOK cells (Fig. 1A). To investigate the effects of miR-148a on SCC-9 cells, cells were transfected with NC, miR-148a mimics or miR-148a inhibitor for 48 h. RT-qPCR analysis was conducted to determine the expression levels of miR-148a. It was demonstrated that miR-148a mimics significantly upregulated the expression of miR-148a in SCC-9 cells compared with the control, whereas miR-148a inhibitor exhibited opposing effects (Fig. 1B and C).

Effects of miR-148a on the viability, migration and invasion of OSCC cells. The viability of SCC-9 cells following transfection was analyzed using an MTT assay. It was demonstrated that miR-148a mimics significantly reduced the viability of cells compared with the control (Fig. 2A). The migration of cells was determined via a scratch-wound assay. Cell migration was markedly reduced following transfection with miR-148a mimics compared with the control (Fig. 2B). Additionally, a Matrigel invasion assay demonstrated that the invasive ability of SCC-9 cells was notably decreased following the overexpression of miR-148a (Fig. 2C). Conversely, transfection with miR-148a inhibitor induced opposing effects on the viability, migration and invasion of SCC-9 cells.

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Luciferase activity in cells transfected with reporters containing the WT, but not the MUT HLA-G 3'-UTR compared with the control (Fig. 4B). The results provided indicated that HLA-G was a direct target of miR-148a. RT-qPCR was conducted to determine the expression levels of HLA-G mRNA in HOK and SCC-9 cells. It was demonstrated that HLA-G was significantly upregulated in SCC-9 cells compared with normal cells (Fig. 4C).

Discussion

It has been reported that miRNAs serve important roles in the regulation of the viability, invasion and migration of cells (28-30). In addition, miRNA dysregulation is involved in the initiation and progression of cancer (31-33). Therefore, miRNAs have received increasing focus as potential therapeutic targets. Previous studies have identified the involvement of various miRNAs in the initiation and progression of oral cancer, including miR-31, miR-139, miR-195, miR-21, miR-221 and miR-455 (34-42). Numerous studies have reported the downregulation of miR-148a in various cancers, including gastric and breast cancers, and esophageal squamous cell carcinoma (22,24), suggesting it exhibits tumor suppressor roles in cancer cells. In the present study, miR-148a was observed to be downregulated in the OSCC cell line, SCC-9 compared with normal cells. Overexpression of miR-148a suppressed the viability, migration and invasive ability of SCC-9 cells. Furthermore, a previous study demonstrated that miR-148a induced the apoptosis of numerous colorectal cancer cell lines, in which Bcl-2 was identified as a direct target (43).

miRNAs can function as oncogenes or tumor suppressors via interactions with specific targets (44,45). HLA-G was identified as a direct target gene of miR-148a. HLA-G is a non-classical HLA-class Ib molecule expressed in EVT cells (8,9). It serves important roles in the establishment of healthy pregnancy; it induces maternal immune tolerance and directly affects the function of trophoblast cells, regulating prefoldin and MMP expression in an autocrine manner (46). Previous studies reported that HLA-G was preferentially expressed in tumor tissues and rarely in adjacent normal tissues, suggesting that it is associated with tumor growth and development (47,48). The findings of the present study revealed that transfection with miR-148a mimics significantly inhibited the expression of HLA-G in OSCC cells, whereas miR-148a inhibitor upregulated HLA-G expression. Additionally, it was demonstrated that miR-148a mimics suppressed the expression of β-catenin, MMP-7 and TCF-4, whereas transfection with miR-148a inhibitor exhibited opposing effects.

In conclusion, miR-148a suppressed the viability, migration and invasion of OSCC cells, potentially via interactions with HLA-G. Thus, targeting the miR-148a/HLA-G interaction may be a novel therapeutic strategy in the treatment of OSCC; however, the present study is only a preliminary investigation into the roles of miR-148a in OSCC. Additional studies are required to determine the expression and role of HLA-G in OSCC tissues, the effects of miR-148a on OSCC in vivo and the association between the expression of miR-148a/HLA-G and the clinical characteristics and prognosis of patients with OSCC.
Figure 3. miR-148a downregulates the expression of invasion and migration-associated proteins in oral squamous carcinoma cells. (A) Representative western blot of HLA-G, β-catenin, MMP-7 and TCF-4 expression in Scc-9 cells following transfection with miR-148a mimics. mRNA expression of (B) HLA-G, (C) β-catenin, (D) MMP-7 and (E) TCF-4 in Scc-9 cells following transfection with miR-148a mimics, as determined by RT-qPCR. (F) Representative western blot of HLA-G, β-catenin, MMP-7 and TCF-4 expression in Scc-9 cells following transfection with miR-148a inhibitor. mRNA expression of (G) HLA-G, (H) β-catenin, (I) MMP-7 and (J) TCF-4 in Scc-9 cells following transfection with miR-148a inhibitor, as determined by RT-qPCR. Data are presented as the mean ± standard deviation. Control, non-transfected cells; HLA-G, human leukocyte antigen-G; miR-148a, microRNA-148a; inhibitor, miR-148a inhibitor; mimics, miR-148a mimics; MMP-7, metalloproteinase-7; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TCF-4, T-cell factor-4. **P<0.01 vs. NC.

Figure 4. Hla-G is direct target of miR-148a. (A) miR-148a binding site in the 3'-UTR of Hla-G, as predicted by TargetScan. (B) Luciferase activity of Scc-9 cells co-transfected with luciferase reporter plasmids containing the WT or mutant 3'-UTR of Hla-G and miR-148a mimics or NC. **P<0.01 vs. NC. (C) Relative mRNA expression of HLA-G in cancer (Scc-9) and normal cells (human oral keratinocytes). **P<0.01 vs. normal cells). Data are presented as the mean ± standard deviation. HLA-G, human leukocyte antigen-G; miR-148a, microRNA-148a; NC, negative control; 3'-UTR, 3'-untranslated region; WT, wild-type.
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Availability of data and materials

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

Authors' contributions

YZ designed the present study. YZ and XJ collected and analyzed the data. JW analyzed the data and drafted the manuscript. All authors interpreted the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The manuscript. All authors interpreted the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YZ designed the present study, YZ and XJ collected and analyzed the data. JW analyzed the data and drafted the manuscript. All authors interpreted the data and wrote the manuscript. All authors read and approved the final version of the manuscript.
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