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MiR-376c-3p regulates the proliferation, invasion, migration, cell cycle and apoptosis of human oral squamous cancer cells by suppressing HOXB7

Kai Wang, Jun Jin, Tengxiao Ma, Hongfeng Zhai*

Department of Plastic Surgery, Henan Provincial People's Hospital, Zhengzhou, 450003, Henan, China

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ABSTRACT

Purpose: To test the influence of miR-376c-3p on the proliferation, invasion, migration, cell cycle and apoptosis of human oral squamous cancer cells (OSCC) and the relevant mechanism.
Methods: We applied qRT-PCR and Western blot to compare the expression level of miR-376c-3p and HOXB7 in SCC-4, SCC-9, SCC-15, SCC-25 OSCC cell lines and 49 paired OSCC and normal oral epithelial tissue specimens were included in our present study. Also we analyzed the relative relationship of expression level between miR-376c-3p and HOXB7 in cancer tissues. Luciferase assay was used to confirm the target relationship between miR-376c-3p and HOXB7. Besides, MTT, Transwell, wound healing, colony formation and flow cytometer experiments were applied to evaluate the proliferation, cell viability, apoptosis, invasion and migration of transfected OSCC.
Results: MiR-376c-3p was down-regulated while HOXB7 was up-regulated in OSCC tissues and cells than the normal ones. MiR-376c-3p directly targeted HOXB7 and reduced the expression of HOXB7. Overexpression of miR-376c-3p attenuated proliferation of SCC-9, SCC-15, SCC-24 and SCC-25 cells. Moreover, miR-376c-3p suppressed proliferation, viability, migration and invasion and induced G1/G0 arrest and cell apoptosis of SCC-25 cells. Besides, overexpression of HOXB7 efficiently abrogates these influences caused by overexpression of miR-376c-3p.
Conclusion: MiR-376c-3p suppresses the fission, proliferation, migration and invasion and induces cell apoptosis of OSCC via targeting HOXB7.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most prevalent cancer with a mortality rate of more than 50% annually, accounting for approximately 90% of all oral malignancies [1,2]. Most OSCC patients died from tumor metastasis and recurrence, indicating that the invasion ability is firmly correlated with the poor prognosis [3,4]. Therefore, the evaluation of new molecular targets in the carcinogenesis and tumor progression of OSCC is urgently required to develop therapeutic strategies for OSCC [5].

MiRNAs are a class of small noncoding RNAs that regulate gene expression at the post-transcriptional level by suppressing ribosome function or promoting the degradation of target mRNAs [5]. The upregulation of oncogenic miRNAs and the down-regulation of tumor suppressor miRNAs can both exert crucial effects in the carcinogenesis and tumor progression [6]. Mi*R*-376c is a member of the mi*R*-379/miR-656 cluster which is a large miRNA cluster located in the imprinted DLK-DIO3 region of human chromosome 14 [7]. Iwaki et al. indicated that the under-expression of mi*R*-376c was reported to accelerate EGF-mediated migration in the HuCCT1 human intrahepatic cholangiocarcinoma cell line [8]. Ye et al. indicated that mi*R*-376c was crucial in regulating cell proliferation, apoptosis, and invasion in ovarian cancer by targeting activin receptor-like kinase 7 (ALK7) [9,10]. As far as we know, mi*R*-376c has always been shown to serve as a tumor suppressor and closely correlate with the metastases and recrudescence of malignant tumors including head and neck squamous cell carcinoma, prostate cancer, lung cancer etc. [1–3]. However, the functions of mi*R*-376c for regulating OSCC and related molecular mechanism remain unknown.

HOX genes belong to a superfamily of homeobox genes that encodes transcription factors with important effects on modulating primary cellular processes, including cell recognition, growth

Abbreviations: OSCC, oral squamous cancer cells; ALK7, activin receptor-like kinase 7; POM, polyformaldehyde; miRNA, microRNA; OSCC, oral squamous cell carcinoma.

* Corresponding author at: Department of Plastic Surgery, Henan Provincial People's Hospital, No.7 Weiwu Road, Zhengzhou, 450003, Henan, China.
E-mail address: kgsuhaiyang@163.com (H. Zhai).

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and differentiation, which was recently discovered to be regulated by miRNAs [11]. 39 HOX genes have been recognized and they are divided into 4 paralogous clusters, including HOXA, HOXB, HOXC and HOXD on autosomal chromosomes [12–15]. Genes of the HOX family are highly expressed in leukemias, melanomas and in breast, ovarian, cervical, esophageal and prostate cancers [16–22]. In addition, the aberrant expression of HOX genes was observed in oral squamous cell carcinomas (OSCC) [23]. HOXB7, a member of the HOX family of homeodomain transcription factors, is a critical developmental regulator which influences the proliferation and survival of progenitor cells. However, its upregulation has been reported in several malignancies which contributed to tumorigenesis. For example, Coletta et al. have previously shown that HOXB7 is overexpressed in OSCC, and its increased expression in the HaCAT human epithelial cell line significantly promoted cell proliferation [24]. Importantly, they found that the immune-expression of HOXB7 tended to be correlated with patient survival in a small set of 35 OSCC cohorts [24]. Apart from regulating cell proliferation, HOXB7 influences numerous other cellular processes, including cell invasion, DNA repair, metastasis, and angiogenesis, which were associated with poor survival of patients with breast cancer and colorectal cancer [25–27].

In the present study, we aimed to clarify the significance of mi*R*-376c-3p and HOXB7 in the treatment of OSCC. We analyzed the expression levels of mi*R*-376c-3p and HOXB7 in normal oral mucosa and OSCC epithelial cells, and verified the relationship between the two cytokines. After manipulating the expression of mi*R*-376c-3p and HOXB7 via cell transfection, we confirmed the tumor-suppressor role of mi*R*-376c-3p on OCSS cell proliferation and metastases, and the tumor-suppressing effect of mi*R*-376c-3p could be efficiently abrogated by overexpression of HOXB7. Taken together, we have assessed the role of mi*R*-376c-3p/HOXB7 axis for regulating the carcinogenesis and tumor progression of OSCC and rendered a new light on the treatment of OCSS.

2. Materials and methods

2.1. Tissue specimens

Forty-nine human OSCC tissue samples were obtained from Henan Provincial People's Hospital. All the patients were diagnosed as oral squamous cancer for the first time and without treatment of radio or chemical therapy before the enrollment. There were 29 males and 20 females with an overall average age of 60 ± 17 yr. The informed consent was signed by all participants. This study had been approved by the Ethics Committee of Henan Provincial People's Hospital.

2.2. Cell culture and transfection

2.2.1. Human embryo kidney 293T cells and oral squamous cancer cell lines

Human embryo 293T cells and SCC-4, SCC-9, SCC-15 and SCC-25 oral squamous cancer cell lines were all purchased from ATCC (American type culture collection). They were all cultured in DMEM containing 10% FBS in 5% CO_2 at 37 °C for 2–3 days.

2.2.2. Primary culture of normal oral epithelial cells

Digestive juice containing 0.25% dispase was applied to digest normal tissues. Then the tissues were washed by PBS. Mixed digestive juice (0.25% trypsin: 0.02%EDTA = 1:1) was added and then we vibrated the mixture to accelerate the digesting process. The cells were suspended and centrifuged.

2.2.3. Cell transfection

Lipofectamine™2000 liposome (Invitrogen, USA) and pWPI lentiviral vector system (Cambrudge, MA, US) were used to carry out the transfection of mi*R*-376c-3p mimics, mimics control and *HOXB7* cDNA according to the manufacturer's instrument. Mi*R*-376c-3p mimics, mimics control, *HOXB7* cDNA were synthesized by Sangon Biotech, China.

2.3. QRT-PCR (quantitative real-time PCR)

QRT-PCR was applied to determine the expression level of miRNA and mRNA. The total RNA was extracted using TRIZOL (Invitrogen, USA). RT kit (Fermentas, USA) and PCR kit (Invitrogen, USA) were used for the PCR. The qRT-PCR amplification procedure was as follows: pre-denaturation at 95 °C for 10s, annealing at 55 °C for 45s, and the cycle was carried out for 40 times. U6 and GAPDH were applied as internal controls for miRNA and mRNA. We used $2^{-\Delta\Delta C_t}$ method to calculate the relative expression of miRNA and mRNA. Primer sequences are listed in Table 1.

2.4. Western blot

Total proteins were extracted using RIPA reagents according to the introduction (Shanghai Gefan Biotechnology Co., Ltd.) and separated by 10% SDS-PAM. Electrophoresis was carried out under a constant voltage of 110 V. The separated proteins were then electro-transferred to the PVDF membranes. The membranes were incubated in 5% nonfat milk at room temperature. HOXB7 primary antibody (1:1000), GADPH primary antibody (1:800) were incubated with the membranes overnight at 4 °C. HRP (horseradish peroxidase) labeled second antibodies (1:900) (the mentioned antibodies were all purchased from Abcam) were also added and incubated for 2 h at 37 °C. ECL was applied to develop the film/membrane.

2.5. Dual luciferase reporter gene assay

PmirGLO—HOXB7 3′ UTR-wt vectors were obtained by connecting the *HOXB7* 3′UTR sequence which contains the mi*R*-376c-3p binding sites to pmirGLO plasmids. Similaryly, pmirGLO—HOXB7 3′-UTR-mut vectors were obtained by connecting the mutated HOXB7 3′UTR sequence (Sangon Biotech, China) to pmirGLO plasmids. The pmirGLO—HOXB7 3′UTR-wt plasmid or pmirGLO—HOXB7 3′UTR-mut plasmid, Renilla Luciferase internal reference plasmid and mi*R*-376c-3p mimics or mimics-NC were transfected into human embryo kidney 293T cells. Meanwhile we set a negative control group, in which the cells were transfected pmirGLO-antisense sequences vectors. All these procedures were carried out according to the introduction of lipofectamine™2000 kit. After 8h's cell transfection, we applied Dual-Luciferase® Reporter Assay System (Promega, USA) to measure the luciferase activity. The results were presented as the ratio of firefly luciferase activity to renilla luciferase activity. We utilized Δluciferase

Table 1
Primer sequences for qRT-PCR.

		Primer
Mi*R*-376c-3p	Forward	5′-ACACTCCAGCTGGGUAUGGCUUUAAGGUGCA-3′
U6	Forward	5′ −CTCGCTTCGGCAGCACA-3
	Reverse	5′-AACGCTTCACGAATTTGCGT-3′
HOXB7	Forward	5′-GGAAGCAGCAGCAAGAACC-3′
	Reverse	5′-CCTCTTCGGCAGACTCTTGTGA-3′
GAPDH	Forward	5′-ACAACTTTGGTATCGTGGAAGG-3′
	Reverse	5′-GCCATCACGCCACAGTTTC-3′