Original Paper

MicroRNA-375 Inhibits Growth and Enhances Radiosensitivity in Oral Squamous Cell Carcinoma by Targeting Insulin Like Growth Factor 1 Receptor

Bin Zhang^a Ying Li^b Daorong Hou^c Quan Shi^a Shuo Yang^a Qihong Li^d

^aDepartment of Stomatology, Chinese PLA General Hospital, Beijing, ^bDepartment of Stomatology, General Hospital of the Air Force of Chinese PLA, Beijing, ^cAnimal Core Facility of Nanjing Medical University, Nanjing, ^dDepartment of Stomatology, Affiliated Hospital of Academy Military Medical Science, Beijing, China

Key Words

miR-375 • Oral squamous cell carcinoma • Insulin like growth factor 1 receptor • Proliferation • Radiosensitivity

Abstract

Background: MicroRNAs (miRNAs) have emerged as key players in various human biological processes, including tumorigenesis. Here, we investigated the roles of miR-375 in the pathogenesis of oral squamous cell carcinoma (OSCC). **Methods:** We performed quantitative real-time PCR (qRT-PCR) to detect miR-375 expression in OSCC tissues and corresponding normal oral epithelial tissues and analyze the correlation of miR-375 expression with OSCC metastasis and patient's survival. Then, the effects of miR-375 expression on proliferation, cell cycle, apoptosis and radiosensitivity in OSCC cells were determined by using MTT, flow cytometry and clonogenic survival assays. A dual-luciferase reporter assay was performed to test whether miR-375 binds to the 3'-untranslated region (3'-UTR) of target mRNA. **Results:** The expression level of miR-375 in OSCC tissues was significantly lower than that in normal oral epithelial tissues, and low miR-375 expression was correlated with higher incidence of lymph node metastasis and poor survival of OSCC patients. Upregulation of miR-375 significantly inhibits growth, induces cell cycle arrest in G₀/G₁ phase, increases apoptosis and enhances radiosensitivity in OSCC cells. Analysis of luciferase activity demonstrated that miR-375 binds to the 3'-UTR of insulin like growth factor 1 receptor (IGF-1R). Small interfering RNA (shRNA)-mediated IGF-1R knockdown mimics the effects of miR-375 upregulation, while overexpression of IGF-1R partially reverses those effects in OSCC cells. **Conclusion:** It was obviously demonstrated that miRNA-375 inhibits growth and enhances radiosensitivity in OSCC cells by targeting IGF-1R, suggesting that miR-375 may be a potential therapeutic target for OSCC patients.

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B. Zhang, Y. Li and D. Hou contributed equally to this work.

Bin Zhang, MD,
and Qihong Li, MD

Department of Stomatology, Chinese PLA General Hospital, Beijing, 100853, (China)
and Department of Stomatology, Affiliated Hospital of Academy Military Medical
Science, Beijing 100071, (China); E-Mail zhangbinfmm@163.com/ liqihong@126.com

Introduction

Oral squamous cell carcinoma (OSCC), the most common cancer of the head and neck, is one of the leading causes of oral cancer-related death around the world due to poor prognosis [1]. Much progress made in OSCC therapy by surgery, chemo- or radiotherapy, but the 5-year survival rate of OSCC still remains under 60%. Similar to other tumors, the pathogenesis of OSCC is a complicated process involving multiple events and steps [2]. Some molecular pathogenesis studies have been undertaken successfully, however the carcinogenic mechanism of OSCC still remains poorly understood. Thus, it is urgent for us to understand the molecular bases of OSCC development to identify potential molecular therapeutic targets for the treatment of OSCC.

MicroRNAs (miRNAs), a class of small non-coding RNAs, negatively regulate gene expression at post-transcription level through their base-pairing with the 3'-untranslated region (3'-UTR) [3, 4]. Accumulating evidence has indicated that deregulation of miRNAs plays a critical role in a variety of physiological and biological processes and many other diseases, including cancer [5-7]. Some dysregulated miRNAs have been reported to be correlated with OSCC development. By analysis of the miRNA expression profiles of OSCC tumors and normal oral mucosal samples, Soga' et al showed that OSCC tissues have a unique miRNA profile pattern when compared with that in normal tissues [8]. In another study, Manikandan' et al identified 46 miRNAs that were differentially expressed in OSCC [9]. Likewise, Siow' et al identified 15 upregulated and 4 downregulated miRNAs in OSCC as compared with normal tissues and miR-375 was validated as the most down-regulated miRNA [10]. Yoon and his colleagues investigated the prognostic value of miR-375 and miR-214-3p in early stage OSCC, and testified the model consisting of a two-miR panel with age and gender may be useful in prognostic significance of early stage OSCC patients [11]. Shi' et al also testified the potential involvement of miR-375 in the premalignant progression of OSCC [12]. Although above results clearly indicated that reduced miR-375 may contribute to OSCC development, the effect of miR-375 on phenotypes of OSCC cells is unclear and needs to be further elucidated.

In this study, our results indicated that miR-375 is downregulated in OSCC tissues compared with adjacent normal oral epithelial tissues. Also, reduced miR-375 is observed to be correlated with poorer prognosis of OSCC patients. Upregulation of miR-375 significantly inhibits growth and enhances radiosensitivity in OSCC cells by targeting IGF-1R. Our findings indicate that targeting miR-375 may be utilized as a potential strategy for radiosensitization of human OSCCs.

Materials and Methods

Tumor tissues

A total of 44 paired of resected OSCC and corresponding normal oral epithelial tissues were collected from patients who were diagnosed at the Department of Oral and Maxillofacial Surgery in Jiangsu Province and Nanjing oral medical hospital from 2008 to 2010. Ethical approval was obtained from the hospital and fully informed consent from all patients prior to sample collection. None of all patients had received chemotherapy or radiotherapy before surgery. All tissue samples were confirmed by pathological examinations. Fresh frozen tissues were stored in liquid nitrogen until use. Clinical parameters, including pathological features and TNM stage, were retrospectively collected by reviewing patients' charts. This study was approved by the Review Board of Hospital Ethics Committee

Cell culture

The human OSCC cell line (SCC-4) was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO-BRL) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Quantitative real-time PCR (qRT-PCR) assay

Total RNA was extracted from the tissues or cultured cells using Trizol (Invitrogen, CA, USA) following the manufacturer's instruction and reverse transcribed using Taqma™ microRNA reverse transcription kit and subjected to real-time PCR using TaqMan™ MicroRNA Assay kit (Applied Biosystems, USA) according to the manufacturer's instructions as described previously [13]. Reactions were performed using Stratagene Mx3000 instrument in triplicate. The expression of miRNA was defined based on the threshold cycle (Ct), and relative expression levels were calculated as $2^{-[(Ct \text{ of miR-375}) - (Ct \text{ of U6})]}$ after normalization with reference to expression of U6 small nuclear RNA.

Transfection of oligonucleotides or plasmids

All miR-375/mimics (or miR-NC/mimics) or miR-375/inhibitor (or miR-NC/inhibitor) were all chemically synthesized by GenePharma (Shanghai, China). For IGF-1R interfering, shRNA targeting IGF-1R (shRNA-IGF-1R) (5'-CUGACUACAGGGAUCUCAU-3') and negative control shRNA (shRNA-control) (5'-GAUCUACGGCAGUCUAUCA-3') were inserted into pLMP vector, which were named pLMP/shRNA-IGF-1R or pLMP/shRNA-control, respectively. The plasmid vector (pcDNA/IGF-1R) expressing open-reading frame of IGF-1R was successfully constructed. For transient transfection, SCC-4 cells were plated in 6-well plates and transfected with 100 nM of miR-375/mimics (or miR-NC/mimics) or miR-375/inhibitor (or miR-NC/inhibitor) using Lipofectamine 2000 (Invitrogen, Long Island, NY) according to the manufacturer's protocol. For stable knockdown of IGF-1R, SCC-4 cells were transfected with pLMP/shRNA-IGF-1R or pLMP/shRNA-control using Lipofectamine 2000 and were selected with 5.0 µg/mL of puromycin (Sigma, USA), which were named SCC-4/shRNA-IGF-1R or SCC-4/shRNA-control, respectively.

Western blot assay

Proteins were extracted from the fresh tissues and cultured cells with a protein extraction kit (KeyGEN, Nanjing) and separated in 10% SDS polyacrylamide gels, electrophoretically transferred to polyvinylidene difluoride membranes (Roche), then detected with anti-human IGF-1R and GAPDH antibodies (Santa Cruz, Dallas, TX, USA). Protein loading was estimated using mouse anti-GAPDH monoclonal antibody. Lab Works™ Image Acquisition and Analysis Software (UVP, Upland, CA, USA) were used to quantify band intensities.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The cell proliferation was detected using MTT assay as described previously [14]. Briefly, the transfected SCC-4 cells were seeded in a 96-well plate. The plates were incubated for 24, 48, 72, 96 or 120 hours, and each well was added with 25 µL of MTT (10 mg/mL) and incubated for 4 h. Then, the supernatant was removed, and 150 µL of dimethyl sulfoxide was added to each well (Sigma, USA). After an overnight incubation, absorbance at 490nm was measured using a microplate reader (Bio-Rad, CA, USA).

Clonogenic survival assay

The clonogenic survival assay was performed as described previously [15]. Briefly, the cells were seeded in triplicate at limiting dilutions in 6-well plates for about 24 hours until attached. 24 hours later, the cells were irradiated with different doses of X-ray radiation (0, 2.0, 4.0, 6.0, and 8.0 Gy), respectively. The medium was replaced with a fresh one 24 hours after irradiation. After 7 days of incubation, the colonies were fixed with methanol, stained with 0.5% crystal violet in absolute ethanol and colonies with ≥50 cells were counted under dissection microscope. In each irradiation dose group, surviving fraction of cells was calculated as plating efficiency of the irradiated cells divided by the plating efficiency of the irradiated cells by that of mock control.

Flow cytometric detection of cell cycle and apoptosis

Flow cytometry assay was performed to detect the changes of cell cycle and apoptosis described previously [16]. In brief, the transfected cells were fixed with 70% ethanol and treated with 1 mg/mL RNase at 37°C for 30 min. Then, the cells were stained with propidium iodide (PI; 50 µg/mL; Sigma) at 4°C for 30 min. Subsequently, the cell cycle and apoptosis were analyzed with a FACS flow cytometer (BD Biosciences, CA, USA).

Dual luciferase activity assay

Dual luciferase activity assay was performed as described previously [17]. In brief, The luciferase-3'-UTR-wild-type (wt) reporter (pGL3-IGF-1R/3'-UTR-wt) or luciferase-3'-UTR-mutant (mut) (pGL3-IGF-1R/3'-UTR-mut) plasmids were prepared by inserting the IGF-1R/3'-UTR-wt carrying a putative miR-375 binding site or its mutant sequence into the pGL3-control plasmid (Promega, Madison, WI, USA). SCC-4 cells were seeded in a 48-well plate and co-transfected with miR-375/mimics or miR-NC/mimics, and pGL3 firefly luciferase vectors containing wild-type or mutant IGF-1R/3'-UTR sequence using Lipofectamine 2000. Co-transfected with two days post-transfection, the cells were collected and lysed for measurement of luciferase activities using the Dual-Luciferase Assay kit (Promega, Madison, WI) according the manufacturer's protocol.

Statistical analysis

All statistical analyses were performed using the SPSS 17.0 statistical software package (SPSS, Inc., Chicago, IL, USA). Experimental data were expressed as the mean±SEM. The differences between groups were analyzed using a Student *t* test when only 2 groups or 1-way analysis of variance when more than 2 groups were compared. Bivariate correlations were calculated by Spearman's Rank Correlation Coefficients. Kaplan-Meier method was used to draw the survival curve, and log-rank test was used for survival analysis. A *P* value of less than 0.05 was considered statistically significant.

Results

Reduced miR-375 is observed to be correlated with tumor progression and poor prognosis of OSCC patients

To explore the relationship between miR-375 expression and OSCC, the expression of miR-375 was detected in 44 paired of OSCC tissues and corresponding normal oral epithelial tissues using qRT-PCR. As shown in Fig. 1A, the expression level of miR-375 was significantly reduced in OSCC tissues compared with normal oral epithelial tissues ($P<0.001$). Also, the expression of miR-375 was observed to be downregulated in OSCC tissues with lymph node metastasis ($n=18$) compared with tissues without lymph node metastasis ($n=26$) ($P<0.001$; Fig. 1B). Furthermore, the OSCC patients with low miR-375 expression level ($n=19$) had poorer overall survival rate than those patients with high miR-375 level ($n=25$) ($P=0.0076$; Fig. 1C). These results suggested that reduced miR-375 may play a role in OSCC development.

Fig. 1. Expression of miR-375 in OSCC tissues and its correlation with lymph node metastasis and survival of OSCC patients. (A) qRT-PCR detection of miR-375 expression in 44 paired of OSCC tissues and corresponding normal oral epithelial tissues ($P<0.001$). U6 was used as the endogenous control. (B) qRT-PCR detection of miR-375 expression in OSCC tissues with lymph node metastasis ($n=26$) and OSCC tissues without lymph node metastasis ($n=18$) ($P<0.001$). (C) Kaplan-Meier survival curves of 44 OSCC patients divided by miR-375 expression. Log-rank test was used for survival analysis. Experimental data are shown as mean±SD of three independent assays.

Fig. 2. Effect of miR-375 expression on proliferation, cell cycle, apoptosis and radiosensitivity in OSCC cells. (A) qRT-PCR detection of miR-375 expression in SCC-4 cells transiently transfected with miR-375/mimics (or miR-NC/mimics) or miR-375/inhibitor (or miR-NC/inhibitor), respectively. U6 was used as the endogenous control. (B) MTT assay was performed to determine the effect of miR-375 upregulation or downregulation on SCC-4 cell proliferation. (C) Flow cytometry was performed to determine the effect of miR-375 upregulation on cell cycle of SCC-4 cells. (D) Flow cytometry was performed to determine the effect of miR-375 upregulation on apoptosis of SCC-4 cells. (E) Clonogenic survival assay was performed to determine the effect of miR-375 upregulation on radiosensitivity of SCC-4 cells. (F) Clonogenic survival assay was performed to determine the effect of miR-375 downregulation on radiosensitivity of SCC-4 cells. (G) Flow cytometric detection of apoptosis in miR-375/mimics or miR-NC/mimics-transfected SCC-4 cells with irradiation treatment (0.0, 2.0, 4.0, 6.0 or 8.0 Gy). (H) Flow cytometric detection of apoptosis in miR-375/inhibitor or miR-NC/inhibitor-transfected SCC-4 cells with irradiation treatment (0.0, 2.0, 4.0, 6.0 or 8.0 Gy). Experimental data are shown as mean $\pm$ SD of three independent assays. N.S., $P > 0.05$; * $P < 0.05$ and ** $P < 0.01$.

Effects of miR-375 expression on growth, apoptosis and radiosensitivity in OSCC cells

To explore the biological functions of miR-375 in OSCC cells, SCC-4 cell line was transfected with miR-375/mimics (or miR-NC/mimics) or miR-375/inhibitor (or miR-NC/inhibitor), respectively. At 48h after transfection, the transfected cells were collected for

Fig. 3. miR-375 directly regulates IGF-1R by binding to the 3'-UTR of IGF-1R mRNA. (A) The putative target genes and mutant complementary sequences of the 3'-UTR of IGF-1R mRNA. (B) Luciferase reporter assays were performed to detect the luciferase activities of the wild type IGF-1R/3'-UTR (pGL3-IGF-1R/3'-UTR-wt) and mutant type IGF-1R/3'-UTR (pGL3-IGF-1R/3'-UTR-mut) co-transfected with miR-375/mimics or miR-NC/mimics were measured. (C) The expression level of IGF-1R mRNA in SCC-4 cells transfected with miR-375/mimics (or miR-NC/mimics) or miR-375/inhibitor (or miR-NC/inhibitor) was determined by qRT-PCR assay. GAPDH was used as the endogenous control. (D) The expression level of IGF-1R protein in SCC-4 cells transfected with miR-375/mimics (or miR-NC/mimics) or miR-375/inhibitor (or miR-NC/inhibitor) was determined by Western blot assay. GAPDH was used as the endogenous control. Experimental data are shown as mean $\pm$ SD of three independent assays. *N.S.*, $P > 0.05$; * $P < 0.05$ and ** $P < 0.01$.

further experiments. The results of qRT-PCR (Fig. 2A) indicated that the expression level of miR-375 was significantly increased by in miR-375/mimics-transfected SCC-4 cells in comparison with the control group ($P < 0.01$) and the expression level of miR-375 was reduced by in miR-375/inhibitor-transfected SCC-4 cells in comparison with the control group ($P < 0.01$). First, we performed MTT assays to analyze the effect of miR-375 expression on growth of OSCC cells (Fig. 2B). Upregulation of miR-375 significantly reduced growth in SCC-4 cells, while downregulation of miR-375 moderately promoted growth in SCC-4 cells. Then, we performed flow cytometry to detect the changes of cell cycle and apoptosis. Results of cell cycle analysis showed that transfection of miR-375/mimics induced the increased accumulation in G_0/G_1 phase and decreased accumulation in S phase but no obvious changes in G_2/M phase (Fig. 2C). Meanwhile, the apoptotic rate of miR-375/mimics-transfected SCC-4 cells was increased by about 19.32%, compared with the control group ($P < 0.05$; Fig. 2D). Furthermore, we investigated the effect of miR-375 expression on radiosensitivity in OSCC cells. In SCC-4 cells, the survival rate was observed to be reduced in 2.0, 4.0, 6.0 and 8.0-Gy-irradiated SCC-4/miR-375/mimics cells, when compared with SCC-4/miR-NC/mimics cells (Fig. 2E). However, the survival rate in 4.0, 6.0 and 8.0 Gy-irradiated SCC-4/miR-375/inhibitor cells was observed to be increased, in comparison with the control group (Fig. 2F). In addition, after the transfected cells were treated with irradiation (2.0, 4.0, 6.0 or 8.0Gy), the apoptosis was determined. Upregulation of miR-375 significantly enhanced irradiation-induced apoptosis in SCC-4 cells (Fig. 2G), while downregulation miR-375

reduced irradiation-induced apoptosis in SCC-4 cells (Fig. 2H). These results suggest that upregulation of miR-375 inhibits growth, induces cell cycle arrest in G_0/G_1 phase, increases apoptosis and enhances radiosensitivity in OSCC cells.

miR-375 directly targets IGF-1R in OSCC cells

To better understand the mechanisms of miR-375 function in OSCC, we performed a bioinformatics (two algorithms: TargetScan and PicTar) search for potential targets of miR-375 and IGF-1R was selected for further analysis. A complementary sequence of miR-375 to the 3'-UTR of IGF-1R mRNA (position: 7423-7429nt) is shown in Fig. 3A. Although IGF-1R has been identified as a direct target of miR-375 in laryngeal squamous cell cancer and cervical cancer [18, 19], whether miR-375 targets IGF-1R to function in OSCC cells is unclear. First, the dual-luciferase reporter assay was performed and the luciferase activity was determined (Fig. 3B). The relative luciferase activity of the luciferase reporter fused to the wild-type of IGF-1R/3'-UTR (IGF-1R/3'-UTR-wt) was significantly reduced ($P<0.01$) when miR-375/mimics was co-transfected into SCC-4 cells. However, the luciferase activity in cells with the reporter gene fused to the mutated-type of IGF-1R/3'-UTR (IGF-1R/3'-UTR-mut) showed no obvious changes ($P>0.05$). Then, qRT-PCR and Western blot assays were performed to analyze the effects of miR-375 expression on IGF-1R mRNA and protein expression in SCC-

Fig. 5. Overexpression of IGF-1R partially reverses the effects of miR-375 upregulation in OSCC cells. (A) qRT-PCR and (B) Western blot detection of IGF-1R mRNA expression in SCC-4 cells transfected with miR-375/mimics (or miR-NC/mimics) or co-transfected with miR-375/mimics and pcDNA/IGF-1R (or pcDNA/control), respectively. GAPDH was used as the endogenous control. (C) MTT assay analysis of proliferation in SCC-4 cells transfected with miR-375/mimics (or miR-NC/mimics) or co-transfected with miR-375/mimics and pcDNA/IGF-1R (or pcDNA/control), respectively. (D) Flow cytometric analysis of apoptosis in SCC-4 cells transfected with miR-375/mimics (or miR-NC/mimics) or co-transfected with miR-375/mimics and pcDNA/IGF-1R (or pcDNA/control), respectively. (E) Clonogenic survival analysis of radiosensitivity in SCC-4 cells transfected with miR-375/mimics (or miR-NC/mimics) or co-transfected with miR-375/mimics and pcDNA/IGF-1R (or pcDNA/control), respectively. (F) Flow cytometric detection of apoptosis in above transfected SCC-4 cells with irradiation treatment (4.0 or 6.0 Gy). Experimental data are shown as mean±SD of three independent assays. *N.S.*, $P>0.05$; * $P<0.05$ and ** $P<0.01$.

4 cells. Transfection of miR-375/mimics significantly decreased the expression of IGF-1R mRNA and protein in SCC-4 cells, while transfection of miR-375/inhibitor increased its mRNA and protein expression in SCC-4 cells (Fig. 3C-D). The data indicated that IGF-1R is a direct target of miR-375 in OSCC cells.

miR-375 functions in OSCC cells partially by targeting IGF-1R

We further confirm that IGF-1R is a functional target of miR-375 in OSCC cells. First, shRNA vector targeting IGF-1R or control vector was stably transfected into SCC-4 cells, respectively. The results of qRT-PCR and Western blot assays confirmed the downregulation of IGF-1R in SCC-4/shRNA-IGF-1R cells in comparison with the control group (Fig. 4A). Functional assays indicated that shRNA-mediated IGF-1R downregulation significantly reduced the capacities of growth, induced cell arrest in G_0/G_1 phase and enhanced apoptosis in SCC-4 cells (Fig. 4B-D). Moreover, the effect of IGF-1R downregulation on radiosensitivity

Fig. 6. miR-375 and IGF-1R are inversely correlated in OSCC tissues. (A) qRT-PCR detection of IGF-1R mRNA expression in 44 paired of OSCC tissues and corresponding normal oral epithelial tissues ($P=0.004$). GAPDH was used as the endogenous control. (B) qRT-PCR detection of IGF-1R mRNA expression in OSCC tissues with lymph node metastasis ($n=26$) and OSCC tissues without lymph node metastasis ($n=18$) ($P=0.012$). (C) A scatter diagram shows an inverse correlation between miR-375 and IGF-1R mRNA expression in the same set of OSCC tissues ($n=44$; Spearman's correlation analysis, $r=-0.37$; $P<0.001$).

in OSCC cells was also analyzed. Compared with SCC-4/shRNA-control cells, the survival rate was significantly decreased in 2.0, 4.0, 6.0 and 8.0 Gy-irradiated SCC-4/shRNA-IGF-1R cells (Fig. 4E). After those transfected SCC-4 cells were treated with irradiation (2.0, 4.0, 6.0 or 8.0 Gy), the apoptosis was determined. Results showed that IGF-1R downregulation significantly increased irradiation-induced apoptosis in SCC-4 cells (Fig. 4F). These data suggested that silencing of IGF-1R mimics the effects of miR-375 upregulation on growth, apoptosis and radiosensitivity in OSCC cells. Then, we performed IGF-1R rescue experiments. Overexpression of IGF-1R reverses the downregulation of IGF-1R mRNA and protein in SCC-4 cells induced by miR-375/mimics (Fig. 5A-B). Meanwhile, overexpression of IGF-1R partially reverses the effects of miR-375 upregulation on growth and apoptosis in SCC-4 cells (Fig. 5C-D). Furthermore, co-transfection of pcDNA/IGF-1R and miR-375/mimics partially reverses growth inhibition and apoptosis enhancement in miR-375/mimics-transfected SCC-4 cells with irradiation treatment (4.0 and 6.0 Gy) (Fig. 5E-F). From above data, it was concluded that miR-375 functions in OSCC cells, at least partially targeting IGF-1R.

Expression of IGF-1R mRNA inversely correlates with miR-375 expression in OSCC tissues

Then, we performed qRT-PCR to detect the expression of IGF-1R mRNA in 40 paired of OSCC tissues and corresponding normal oral epithelial tissues. It was observed that the expression level of IGF-1R mRNA in OSCC tissues was significantly higher than that in corresponding normal oral epithelial tissues ($P=0.004$; Fig. 6A). Meanwhile, the expression level of IGF-1R mRNA in OSCC tissues with lymph node metastasis ($n=16$) was also higher than that in OSCC tissues without lymph node metastasis ($n=24$) ($P=0.012$; Fig. 6B). Next, we performed a correlation test using the above OSCC tissues, and showed that there was a significant inverse correlation between miR-375 and IGF-1R mRNA ($r=-0.37$, $P<0.001$; Fig. 6C), suggesting that a regulatory relationship between miR-375 and IGF-1R may be present in OSCC.

Discussion

In the present study, we showed that miR-375 is downregulated in OSCC tissues and reduced miR-375 is correlated with higher incidence of lymph node metastasis and poor prognosis of OSCC patients. Also, miR-375 inhibits growth, increases apoptosis and enhances radiosensitivity in OSCC cells, at least partially by targeting IGF-1R. The dysregulation of miR-375/IGF-1R axis might play important roles in OSCC development.

MiR-375 has been shown to function as a tumor suppressor in various human cancers. For example, Lian' et al reported that miR-375 functions as a tumor-suppressor gene in gastric cancer by targeting *recepteur d'Origine Nantais* [20]. Xu and his colleagues showed that miR-375 suppresses human colorectal cancer metastasis by directly acting upon *Frizzled 8*, suggesting that this miRNA may serve as a new therapeutic target to inhibit colorectal cancer metastasis [21]. In another report, miR-375 is also found to inhibit the invasion and metastasis of colorectal cancer via targeting *SP1* and regulating EMT-associated genes [22]. Osako' et al also showed that miR-375 markedly inhibits cancer cell migration and invasion by regulation of *MMP13* by in esophageal squamous cell carcinoma [23]. Jung and his colleagues showed that four anti-cancer drugs (doxorubicin, 5-fluorouracil, trichostatin A and etoposide) could induce the expression of tumor suppressor miR-375 in tongue cancer [24]. This research group also testified that underexpression of tumor suppressor miR-375 contributes to promoting cancerous phenotypes by inducing uncontrolled *CIP2A* expression and extended stability of *MYC* [25]. Meanwhile, they showed that miR-375 activates *p21* and suppresses telomerase activity by coordinately regulating *HPV E6/E7*, *E6AP*, *CIP2A*, and *14-3-3ζ* [26]. Although miR-375 downregulation in oral premalignant lesions is found to be associated with a higher risk of malignant transformation [27], the functions of miR-375 in OSCC development are unclear. Here, we showed that miR-375 is significantly downregulated in OSCC tissues and the level of miR-375 expression could be used as a biomarker to distinguish patients with or without lymph node metastasis. OSCC patients with low miR-375 expression had shorter survival than those with high miR-375 expression. Next, we analyzed the effect of miR-375 expression on growth, cell cycle and apoptosis of OSCC cells. Ectopic expression of miR-375 inhibits growth, induces cell cycle arrest in G_0/G_1 phase and enhances apoptosis in OSCC cells, while downregulation of miR-375 promotes OSCC cell growth. Recently, some researches also show that miR-375 exerts effect on the sensitivity of tumor treatments. For example, miR-375 is reported to be involved in development of chemoresistance of multiple tumor cells, such as prostate cancer, breast cancer, pancreatic cancer and gastric cancer [28-31], suggesting that miR-375 might serve as potential predictive biomarkers to chemotherapy and/or therapeutic targets to overcome chemoresistance in human cancers. Meanwhile, the correlation of miR-375 with radiosensitivity of tumor cells is also reported. For example, Song et al showed that miR-375 modulates radiosensitivity of HR-HPV-positive cervical cancer cells by targeting *UBE3A* through the *p53* pathway [32]. Zhang and his colleagues showed that targeting *MYCN* IRES in *MYCN*-amplified neuroblastoma with miR-375 inhibits tumor growth and sensitizes tumor cells to radiation, implying the development of miR-375 as a novel therapeutic agent to treat *MYCN*-amplified neuroblastoma [33]. In this study, we also analyzed the effect of miR-375 expression on radiosensitivity of OSCC cells, and showed that upregulation of miR-375 significantly enhances the sensitivity of OSCC cells to irradiation treatment by increasing irradiation-induced apoptosis, suggesting that targeting miR-375 might be a strategy for the treatment of radioresistant OSCC.

MiRNAs exert their functions through partially binding the mRNA, so identification of functional mRNA targets of miR-375 is important for understanding the roles of miR-375 in OSCC progression. By bioinformatics, *IGF-1R* gene was identified as a potential target of miR-375. In laryngeal squamous cell cancer and cervical cancer, *IGF-1R* has been testified as a functional target of miR-375 [18, 19]. *IGF-1R* belongs to the insulin receptor (IR) family which includes the IR, *IGF-1R*, *IGF-1R/IR*, and *IGF-2R*. Previous studies have shown that the *IGF-1R* signaling pathway plays a pivotal role in cancer growth, progression, and resistance to

anticancer therapies [34,35]. It is well known that IGF-1R exerts its main actions through the activation of the mitogen-activated protein kinase and phosphoinositide 3-kinase pathways. Also, activation of IGF-1R is reported to be correlated with increased radioresistance by associating to new partners as major vault proteins (BCL-2, BAX, or Ku70/80), which are correlated with radiochemoresistance [36]. A previous study has shown that autocrine production of IGF-2, together with over-expression of IGF-1R, may be important components controlling the proliferation of OSCC cells [37]. Lara and his colleagues showed that low IGF-1R tumour expression was correlated to better long-term survival in patients suffering locally advanced oral carcinoma [38]. Recently, IGF-1R is testified as a target of let-7b and mediates crosstalk between IRS-2/Akt and MAPK pathways to promote proliferation of OSCC [39]. However, whether miR-375 exerts its functions in OSCC by directly targeting IGF-1R is unclear. Here, luciferase assay indicated that miR-375 could bind to the 3'-UTR of IGF-1R mRNA. Additionally, alteration in miR-375 levels affects expression of IGF-1R both mRNA and protein. Most importantly, silencing of IGF-1R mimics the effects of miR-375 upregulation and overexpression of IGF-1R partially rescues the effects of miR-375 upregulation in OSCC cells. Also, there was a significant inverse correlation between miR-375 and IGF-1R mRNA in OSCC tissues. These results demonstrate that miR-375/IGF-1R regulatory axis might play a role in promotion of OSCC progression. As a single microRNA can potentially regulate multiple mRNA targets, whether miR-375 functions in OSCC cells by targeting other mRNAs needs to be elucidated in future study. Also, the mechanisms by which miR-375 and IGFR can affect radiosensitivity of OSCC cells need to be elucidated in future study.

Taken together, our study showed that reduced miR-375 is correlated with OSCC progression and poor survival of patients. Functional experiments indicated that miR-375 inhibits growth, increases apoptosis and enhances radiosensitivity in OSCC cells, at least partially by targeting IGF-1R. Therefore, miR-375 may be a potential therapeutic molecular target for OSCC therapy.

Disclosure Statement

The authors declare that they have no competing interests

References

- 1 Siegel R, Ma J, Zou Z, Jemal A: Cancer statistics, 2014. *CA Cancer J Clin* 2014;64:9-29.
- 2 Dumache R, Rogobete AF, Andreescu N, Puiu M: Genetic and Epigenetic Biomarkers of Molecular Alterations in Oral Carcinogenesis. *Clin Lab* 2015;61:1373-1381.
- 3 Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281-297.
- 4 He L, Hannon GJ: MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 2004;5:522-531.
- 5 Barwari T, Joshi A, Mayr M: MicroRNAs in Cardiovascular Disease. *J Am Coll Cardiol* 2016;68:2577-2584.
- 6 Alipour SD, Adcock IM, Garssen J, Mortaz E, Varahram M, Mirsaedi M, Velayati A: The roles of miRNAs as potential biomarkers in lung diseases. *Eur J Pharmacol* 2016;791:395-404.
- 7 McManus MT: MicroRNAs and cancer. *Semin Cancer Biol* 2003;13:253-258.
- 8 Soga D, Yoshida S, Shioyama S, Miyazaki H, Kondo S, Shintani S: microRNA expression profiles in oral squamous cell carcinoma. *Oncol Rep* 2013;30:579-583.
- 9 Manikandan M, Deva Magendhra Rao AK, Arunkumar G, Manickavasagam M, Rajkumar KS, Rajaraman R, Munirajan AK: Oral squamous cell carcinoma: microRNA expression profiling and integrative analyses for elucidation of tumorigenesis mechanism. *Mol Cancer* 2016;15:28.
- 10 Siow MY, Ng LP, Vincent-Chong VK, Jamaludin M, Abraham MT, Abdul Rahman ZA, Kallarakkal TG, Yang YH, Cheong SC, Zain RB: Dysregulation of miR-31 and miR-375 expression is associated with clinical outcomes in oral carcinoma. *Oral Dis* 2014;20:345-351.

- 11 Yoon AJ, Wang S, Shen J, Robine N, Philipone E, Oster MW, Nam A, Santella RM: Prognostic value of miR-375 and miR-214-3p in early stage oral squamous cell carcinoma. *Am J Transl Res* 2014;6:580-592.
- 12 Shi W, Yang J, Li S, Shan X, Liu X, Hua H, Zhao C, Feng Z, Cai Z, Zhang L, Zhou D: Potential involvement of miR-375 in the premalignant progression of oral squamous cell carcinoma mediated via transcription factor KLF5. *Oncotarget* 2015;6:40172-40185.
- 13 Yao Y, Shao J, Wu J, Zhang Q, Wang J, Xiao D, Huang F: The Functional Variant in the 3'UTR of PTPRT with the Risk of Esophageal Squamous Cell Carcinoma in a Chinese Population. *Cell Physiol Biochem* 2015;36:306-314.
- 14 Liu K, Li L, Rusidanmu A, Wang Y, Lv X: Down-Regulation of MiR-1294 is Related to Dismal Prognosis of Patients with Esophageal Squamous Cell Carcinoma through Elevating C-MYC Expression. *Cell Physiol Biochem* 2015;36:100-110.
- 15 Cui SY, Huang JY, Chen YT, Song HZ, Feng B, Huang GC, Wang R, Chen LB, De W: Let-7c governs the acquisition of chemo- or radioresistance and epithelial-to-mesenchymal transition phenotypes in docetaxel-resistant lung adenocarcinoma. *Mol Cancer Res* 2013;11:699-713.
- 16 Xu CZ, Jiang C, Wu Q, Liu L, Yan X, Shi R: A Feed-Forward Regulatory Loop between HuR and the Long Noncoding RNA HOTAIR Promotes Head and Neck Squamous Cell Carcinoma Progression and Metastasis. *Cell Physiol Biochem* 2016;40:1039-1051.
- 17 Yan S, Jiang H, Fang S, Yin F, Wang Z, Jia Y, Sun X, Wu S, Jiang T, Mao A: MicroRNA-340 Inhibits Esophageal Cancer Cell Growth and Invasion by Targeting Phosphoserine Aminotransferase 1. *Cell Physiol Biochem* 2015;37:375-386.
- 18 Luo J, Wu J, Li Z, Qin H, Wang B, Wong TS, Yang W, Fu QL, Lei W: miR-375 suppresses IGF1R expression and contributes to inhibition of cell progression in laryngeal squamous cell carcinoma. *Biomed Res Int* 2014;2014:374598.
- 19 Yu X, Zhao W, Yang X, Wang Z, Hao M: miR-375 Affects the Proliferation, Invasion, and Apoptosis of HPV16-Positive Human Cervical Cancer Cells by Targeting IGF-1R. *Int J Gynecol Cancer* 2016;26:851-858.
- 20 Lian S, Park JS, Xia Y, Nguyen TT, Joo YE, Kim KK, Kim HK, Jung YD: MicroRNA-375 Functions as a Tumor-Suppressor Gene in Gastric Cancer by Targeting Recepteur d'Origine Nantais. *Int J Mol Sci* 2016;17.
- 21 Xu L, Wen T, Liu Z, Xu F, Yang L, Liu J, Feng G, An G: MicroRNA-375 suppresses human colorectal cancer metastasis by targeting Frizzled 8. *Oncotarget* 2016;7:40644-40656.
- 22 Cui F, Wang S, Lao I, Zhou C, Kong H, Bayaxi N, Li J, Chen Q, Zhu T, Zhu H: miR-375 inhibits the invasion and metastasis of colorectal cancer via targeting SP1 and regulating EMT-associated genes. *Oncol Rep* 2016;36:487-493.
- 23 Osako Y, Seki N, Kita Y, Yonemori K, Koshizuka K, Kurozumi A, Omoto I, Sasaki K, Uchikado Y, Kurahara H, Maemura K, Natsugoe S: Regulation of MMP13 by antitumor microRNA-375 markedly inhibits cancer cell migration and invasion in esophageal squamous cell carcinoma. *Int J Oncol* 2016;49:2255-2264.
- 24 Jung HM, Benarroch Y, Chan EK: Anti-cancer drugs reactivate tumor suppressor miR-375 expression in tongue cancer cells. *J Cell Biochem* 2015;116:836-843.
- 25 Jung HM, Patel RS, Phillips BL, Wang H, Cohen DM, Reinhold WC, Chang LJ, Yang LJ, Chan EK: Tumor suppressor miR-375 regulates MYC expression via repression of CIP2A coding sequence through multiple miRNA-mRNA interactions. *Mol Biol Cell* 2013;24:1638-1648.
- 26 Jung HM, Phillips BL, Chan EK: miR-375 activates p21 and suppresses telomerase activity by coordinately regulating HPV E6/E7, E6AP, CIP2A, and 14-3-3ζ. *Mol Cancer* 2014;13:80.
- 27 Harrandah AM, Fitzpatrick SG, Smith MH, Wang D, Cohen DM, Chan EK: MicroRNA-375 as a biomarker for malignant transformation in oral lesions. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2016;122:743-752.
- 28 Wang Y, Lieberman R, Pan J, Zhang Q, Du M, Zhang P, Nevalainen M, Kohli M, Shenoy NK, Meng H, You M, Wang L: miR-375 induces docetaxel resistance in prostate cancer by targeting SEC23A and YAP1. *Mol Cancer* 2016;15:70.
- 29 Liu SL, Sui YF, Lin MZ: MiR-375 is epigenetically downregulated due to promoter methylation and modulates multi-drug resistance in breast cancer cells via targeting YBX1. *Eur Rev Med Pharmacol Sci* 2016;20:3223-3229.
- 30 Yang D, Yan R, Zhang X, Zhu Z, Wang C, Liang C, Zhang X: Deregulation of MicroRNA-375 inhibits cancer proliferation migration and chemosensitivity in pancreatic cancer through the association of HOXB3. *Am J Transl Res* 2016;8:1551-1559.

- 31 Zhou N, Qu Y, Xu C, Tang Y: Upregulation of microRNA-375 increases the cisplatin-sensitivity of human gastric cancer cells by regulating ERBB2. *Exp Ther Med* 2016;11:625-630.
- 32 Song L, Liu S, Zeng S, Zhang L, Li X: miR-375 Modulates Radiosensitivity of HR-HPV-Positive Cervical Cancer Cells by Targeting UBE3A through the p53 Pathway. *Med Sci Monit* 2015;21:2210-2217.
- 33 Zhang H, Liu T, Yi S, Gu L, Zhou M: Targeting MYCN IRES in MYCN-amplified neuroblastoma with miR-375 inhibits tumor growth and sensitizes tumor cells to radiation. *Mol Oncol* 2015;9:1301-1311.
- 34 Hartog H, Wesseling J, Boezen HM, van der Graaf WT: The insulin-like growth factor 1 receptor in cancer: old focus, new future. *Eur J Cancer* 2007;43:1895-1904.
- 35 Bähr C, Groner B: The insulin like growth factor-1 receptor (IGF-1R) as a drug target: novel approaches to cancer therapy. *Growth Horm IGF Res* 2004;14:287-295.
- 36 Valenciano A, Henríquez-Hernández LA, Moreno M, Lloret M, Lara PC: Role of IGF-1 receptor in radiation response. *Transl Oncol* 2012;5:1-9.
- 37 Brady G, Crean SJ, Naik P, Kapas S: Upregulation of IGF-2 and IGF-1 receptor expression in oral cancer cell lines. *Int J Oncol* 2007;31:875-881.
- 38 Lara PC, Bordón E, Rey A, Moreno M, Lloret M, Henríquez-Hernández LA: IGF-1R expression predicts clinical outcome in patients with locally advanced oral squamous cell carcinoma. *Oral Oncol* 2011;47:615-619.
- 39 Gao L, Wang X, Wang X, Zhang L, Qiang C, Chang S, Ren W, Li S, Yang Y, Tong D, Chen C, Li Z, Song T, Zhi K, Huang C: IGF-1R, a target of let-7b, mediates crosstalk between IRS-2/Akt and MAPK pathways to promote proliferation of oral squamous cell carcinoma. *Oncotarget* 2014;5:2562-2574.