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Original Article

MiR-137 silencing of BRD4 suppresses oral squamous cell carcinoma cells proliferation, migration and invasion

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Abstract: Objective: In order to study the role of miR-137 in oral squamous cell carcinoma (OSCC). Materials and methods: The gene expression level of miR-137 was detected in OSCC tissues and cell lines by using qRT-PCR. OSCC cells were transfected with miR-137 mimics and mimic controls. The proliferation, invasion and migration abilities were measured by MTT, colony formation assay, transwell and wound healing analysis. The targeting gene of miR-137 was measured by western blot, qRT-PCR and luciferase activity assays. Results: We demonstrated that the level of miR-137 was decreased in OSCC tissues when compared with the adjacent normal tissues, it also downregulated in OSCC cell lines. Overexpression of miR-137 suppressed OSCC cells proliferation, invasion and migration. In addition, we demonstrated that bromodomain 4 (BRD4) was a target gene of miR-137. Upregulation of BRD4 can ameliorate the inhibiting effect of miR-137 on tumor cells proliferation and migration. Conclusions: MiR-137 acted as an anticarcinogenic miRNA, partly through targeting BRD4 in oralsquamous cell carcinoma, it would become a therapeutic target for OSCC.

Keywords: miR-137, oral squamous cell carcinoma, BRD4, proliferation, migration, invasion

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies of all malignant lesions of the mouth, it accounts for more than 90% of the mouth tumors and possesses a serious intimidation to human life and healthy [1, 2]. In spite of many advances in treatment, including radical surgery, radiotherapy and neo-adjuvant chemotherapy, OSCC is still associated with a poor prognosis because of its strong local invasion and a high rate of lymph node metastasis, the five year survival rate of patients are about 50% [3-5]. Therefore, it is very critical to investigating the mechanism of invasion and finding a more effective strategy to therapy OSCC.

It has been previously illustrated thatas a family of the bromodomain and extraterminal (BET), bromodomain 4 (BRD4) plays an important role in gene regulation [6]. Abnormal stimulation of the expression of BRD4 gene is related with the oncogenesis of many human cancers. Former researches have reported that BRD4 is dramatically upregulated in a variety of malignant tumors including bladder cancer, non-small cell lung cancer, leukemia and hepatocellular carcinoma [7-10]. Suppressing BRD4 by siRNA and small molecule inhibitors has been proved to be a therapeutic protocol in many cancers [11, 12].

MicroRNAs (miRNAs) are a new class of endogenous, short-length single-stranded, conserved and small RNAs that regulating gene expression through binding to the 3'-untranslated region (3'-UTR) of their target messenger RNAs (mRNAs) [13-15]. A growing body of research has showed that miRNAs play an important role in many biological processes such as cell development, invasion, proliferation, differentiation, metabolism, apoptosis and migration [16-19]. Increasing evidences have demonstrated that dysregulated expression of miRNA is related with tumour initiation, development and cancer death through regulating tumour inhibitor gene or oncogene [19-21]. Early research has shown

that miR-137 played a critical role in the tumour progress [22-25]. For instance, Dang et al. illustrated that miR-137 accelerate methylation in oral lichen planus and oral squamous cell carcinoma [26]. Langevin et al. found that miR-137 is associated with the survival in patients with squamous cell carcinoma [27]. However, the mechanism of miR-137 inhibiting OSCC development was still unknown. In this study, we certified that the level of miR-137 was reduced in OSCC tissues and cells. Upregulation of miR-137 inhibited the OSCC cell proliferation, invasion and migration. What's more, we found that BRD4 is a direct target gene of miR-137. Restoration of BRD4 rescued the OSCC cells proliferation and invasion suppressed by overexpression of miR-137. These findings demonstrated that miR-137 played a repressive role in OSCC by targeting BRD4.

Materials and methods

Clinical tissues and cell lines cultured

Human OSCC tissues and their adjacent noncancer were collected from our hospital. Each patient agreed to participate in this study and was given written informed consent and the research was approved with Declaration of Helsinki and the ethics committee of our hospital. All the tissues were diagnosed and confirmed by pathological examination. SCC4, SCC1, Cal-27 and the normal oral keratinocyte cell lines (NHOK) were purchased from the American Type Culture Collection (ATCC). All the cells were cultivated in the DMEM/F12 medium supplemented with heat-inactivated 10% FBS (GIBCO, Grand Island, NY, USA) and penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively) at 37°C in a humidified atmosphere of 5% CO_2.

Quantitative RT-PCR

Total RNA was extracted from clinical tissues or cell lines by using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was measured by using UV absorbancies at 260 and 280 nm (A260/280). Then the RNA was reverse-transcribed into cDNA using reverse transcription system (Thermo Scientific, CA, USA). The relative expression level of miR-137 and BRD4 was detected by using qRT-PCR according to the manufacturer's instruction. The judgment of primer sequences' specificity was based on dissociation curve, and the expression of miR-137 and BRD4 was measured by $2^{-\Delta\Delta Ct}$ (cycle threshold) method, where GAPDH and U6 were used as acontrol normalize for BRD4 and miR-137 respectively.

Western blot

Protein was isolated by using RIPA buffer which contain a protease inhibitor cocktail and phosphatase inhibitors (Sigma, St. Louis, MO, USA) from clinical samples or OSCC cell lines. The total protein samples were separated by SDS-PAGE gel and then transferred to the polyvinylidene difluoride (PVDF, Millipore, Boston, MA, USA) membranes using the Bio-Rad transfer system. After that the membrane was blocked by 5% milk and then incubated with BRD4 antibody (1:2000; Cell Signaling Technology, CA, USA) and β-actin antibody (1:2000; Cell Signaling Technology, CA, USA). Theprotein signal was detected by an ECL kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions.

Lentivirus production and infection

The miR-137 mimics and miR-137 mimics control were purchased from Gene-Chem (GeneChem, Shanghai, China). Cells were transfected by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's protocols.

Dual-luciferase reporter assay

Cells were incubated in the 24-well plate for 24 h before transfection. The BRD4 3' UTR of BRD4 cDNA including putative site for the miR-137 was synthesized and inserted into the Renilla lucifearse plasmid (Promega, Madison, WI, USA). Cells were co-transfected with miR-137 mimics or mimic control and pGL3-BRD4-3' UTR or MUT 3' UTR for 48 h and detected by the Dual-Luciferase Reporter Assay System (Promega, Madison, USA) according to the manufacturer'sinstruction.

Cell proliferation assay

Cell proliferation was measured by colony formation test and MTT assay. For MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide) assay, cells were treated with miR-383

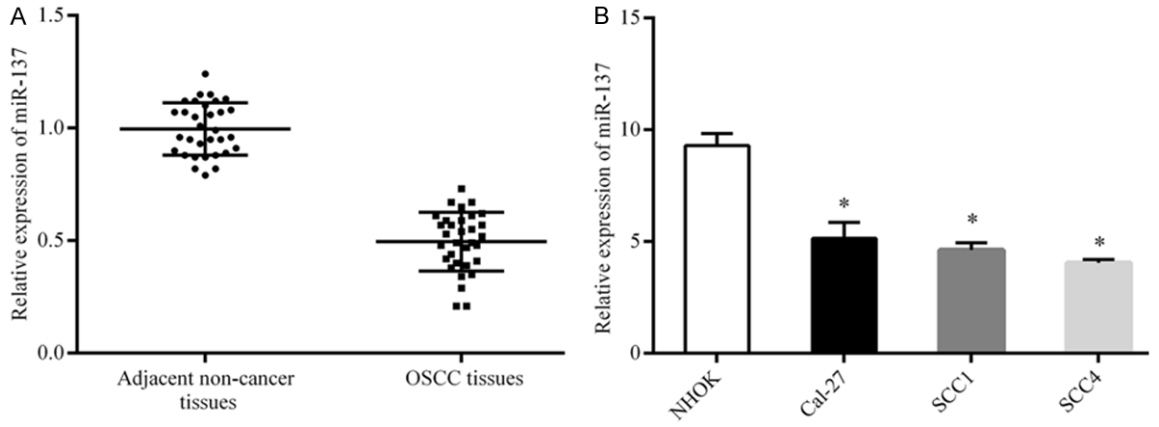

Figure 1. MiR-137 is down-regulated in OSCC tissues and cell lines. A: The level of miR-137 in OSCC tissues are declined when compared with the adjacent non-cancer tissues. B: The expression of miR-137 was decreased in OSCC cell lines ($^{*}P<0.05$ when compared with the NHOK).

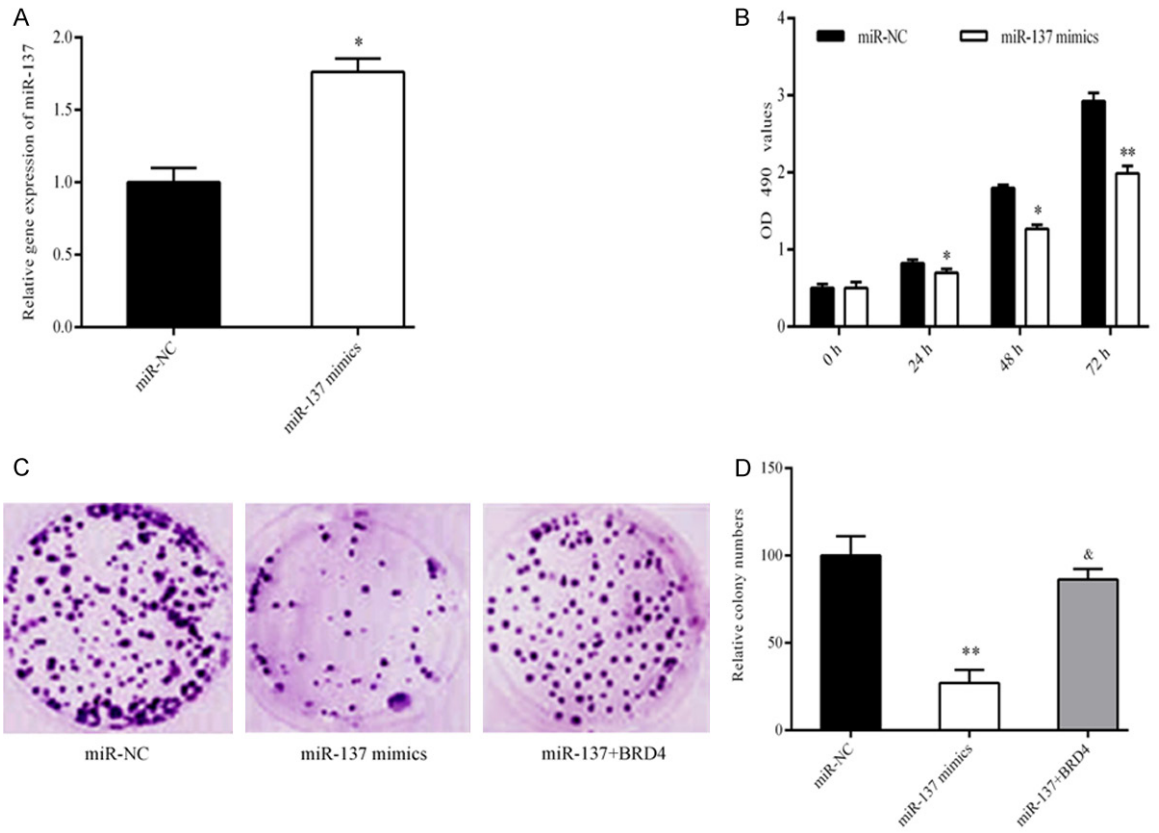

Figure 2. MiR-137 inhibits the proliferation and colony formation of OSCC cells by targeting BRD4. A: The expression of miR-137 was detected in the SCC4 cell aftert reatment with the miR-137 mimics ($^{*}P<0.05$). B: Overexpression of miR-137 inhibited the SCC4 cell proliferation ($^{*}P<0.05$, $^{**}P<0.01$). C, D: Overexpression of miR-137 suppressed the SCC4 cell colony formation while recovered BRD4 could increase it ($^{**}P<0.01$ when compared with the miR-NC, $^{\&}P<0.05$ when compared with the miR-137 mimics).

mimics and miR-383 mimics control then cultured in the 96-well plate. The cells proliferation was measured at 24, 48 and 72 h after transfection. MTT solution was added into the wells and cells were continued to incubate for 4 hours and then added the dimethylsulfoxide. The optical density (OD) value was tested at 490 nm on the microplate reader. For colony

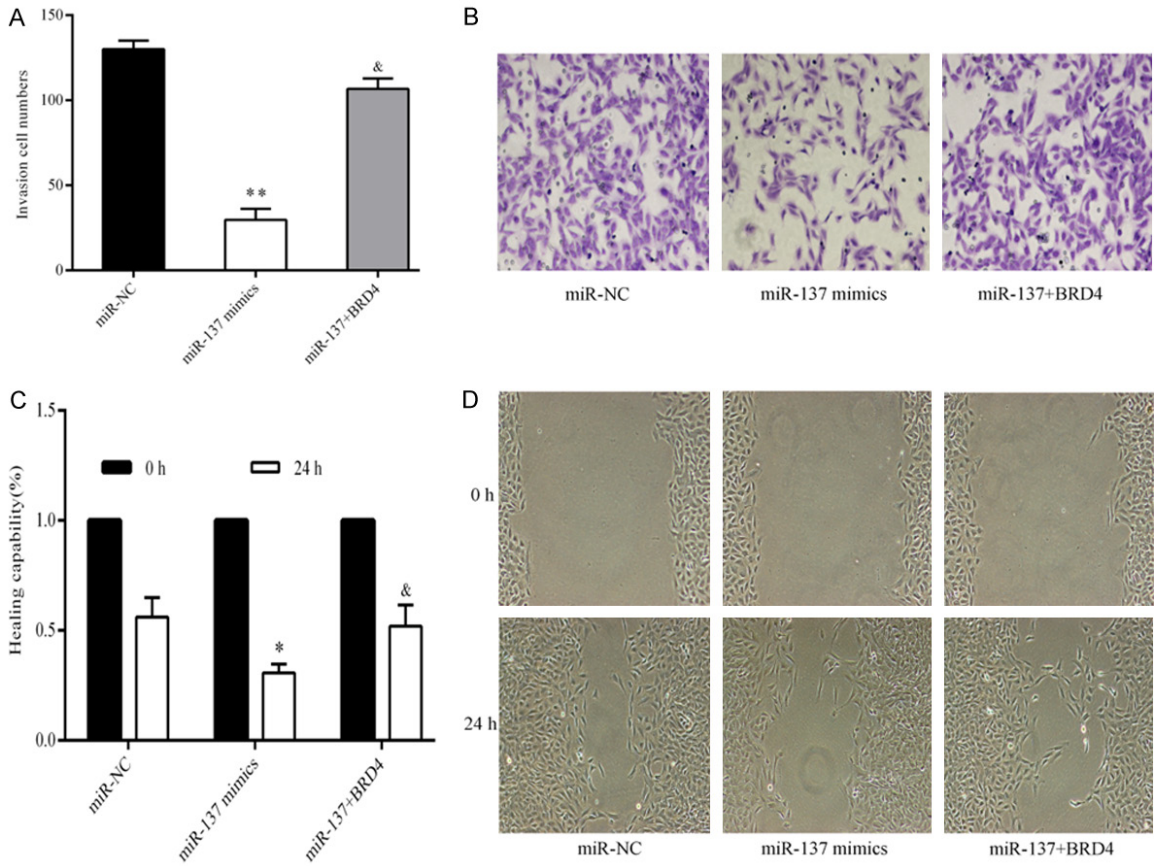

Figure 3. MiR-137 inhibits the invasion and migration of OSCC cells by targeting BRD4. A, B: Invasion assay showed that miR-137 made the invasion cell numbers reduced while recovered BRD4 could increase it ($^{**}P<0.01$ when compared with the miR-NC, $^{\&}P<0.05$ when compared with the miR-137 mimics). C, D: Wound healing assay demonstrated that miR-137 inhibited the SCC4 cells migration while BRD4 could recovery it ($^{*}P<0.05$ when compared with the miR-NC, $^{\&}P<0.05$ when compared with the miR-137 mimics).

formation test, cells were cultivated in the 6-well plate. After cultured for 2 weeks, the colonies were stained with crystal violet and then counted.

Cell migration assay

Cell migration was measured by the wound healing analysis. The cell lines were seeded into 6 well plates and cultured with miR-137 mimics or mimic control. The wounds were created by a sterile pipette tip and washed with PBS for three times. Then the cells continued to culture with medium for another 24 or 48 hours at 37°C. The wound width was measured by using microscope (Nikon, Tokyo, Japan).

Cell invasion assay

For invasion assay, cells were cultivated in the upper chamber with a membrane that was pre-treated with matrigel (100 μg per well, BD Biosciences, San Jose, CA, USA). In the upper chamber, medium without FBS was added while in the lower portion of the chamber, 10% FBS was added. After the cells were incubated for 24 h at 37°C, we carefully removed the cells in the upper chamber. Invaded cells were fixed with 4% formaldehyde, stained with 0.5% crystal violet, and counted under a microscope (Nikon, Tokyo, Japan).

Statistical analysis

All Dates were expressed as the means ± SD (standard deviation). SPSS 19.0 was used to analyze the dates. Difference between the experiment group and the control group was analyzed by using one-way ANOVA test. $P<0.05$ was considered to be statistically significant differences.

Results

MiR-137 expression level was downregulated in OSCC tissues and cell lines

We researched the expression level of miR-137 in OSCC tissues and cell lines. Results showed that the expression level of miR-137 was declined in OSCC tissues when compared with the adjacent normal tissues (**Figure 1A**). And furthermore, we also demonstrated that the level of miR-137 was downregulated in OSCC cell lines than in the NHOK cell (**Figure 1B**).

MiR-137 inhibited OSCC cell proliferation and colony formation

As we discovered the low expression of miR-137 in OSCC tissue and cells, we subsequently examined the effect of miR-137 in the proliferation and colony formation in SCC4 cells.MiR-137 mimics and miR-NC were transfected into SCC4 cell lines and the level of miR-137 was detected by qRT-PCR. The gene expression level of miR-137 in the mimics group was significantly overexpressed when compared with the mimics control (**Figure 2A**). The results demonstrated that the level of miR-137 can be regulated. MTT assays showed that overexpression of miR-137 inhibited SCC4 proliferation (**Figure 2B**). Furthermore, upregualtion of miR-137 suppressed SCC4 cell colony formation (**Figure 2C**, **2D**).

MiR-137 inhibited OSCC cell invasion and migration

The transwell invasion assay demonstrated that increased the level of miR-137 dramatically reduced the cells invaded when compared with the cells transfected with mimics control (**Figure 3A**, **3B**). In addition, the wound-healing assays illustrated a similar tendency (**Figure 3C**, **3D**).

MiR-137 target BRD4 in OSCC cell

We used targetScan found that BRD4 was a direct target of miR-137 (**Figure 4A**). In order to confirm BRD4 was the target of miR-137, BRD4 wild-type (WT) or mutant 3'-UTR was subcloned into a luciferase reporter vector and co-transfected with miR-137 mimics or mimics control into SCC4 cells, results showed that in SCC4 cell lines the luciferase activity of BRD4 WT 3'-UTR was dramatically suppressed by miR-137 but had no influence on the mutant (**Figure 4B**). Overexpression of miR-137 suppressed the mRNA and protein level of BRD4 in SCC4 cell (**Figure 4C**, **4D**). Moreover, the expression of BRD4 was recovery after treatment with BRD4 (**Figure 4E**, **4F**), restoration of BRD4 could reverse the proliferation, migration and invasion of SCC4 cells inhibited by upregulation miR-137 (**Figures 2**, **3**).

Discussion

More and more studies have shown that miRNAs can be act as a tumor regulator, either as a cancer suppressor or anoncogene [28, 29]. For instance, Shen et al. [30] discovered that the expression level of miR-137 was decreased in lung cancer tissues. They illustrated that upregulation of miR-137 restrained lung cancer cells proliferation, migration and invasion by controlling the expression of nuclear casein kinase and cyclin-dependent kinase substrate1 (NUCKS1). Another study showed that the level of miR-137 was reduced in the papillary thyroid carcinoma tissues. Upregulation of miR-137 inhibitedthe papillary thyroid carcinoma cell colony formation, proliferation, invasion and migration through inhibiting the level of C-X-C motif chemokine 12 (CXCL12) [31].

In this study, we found that the level of miR-137 was decreased in OSCC tissues when compared with the adjacent normal tissues. In addition, we also demonstrated that miR-137 was downregulated in OSCC cell lines compared to the NHOK cells. Furthermore, we studied the roles of miR-137 in OSCC cells. First we confirmed that the expression level of miR-137 can be regulated in OSCC cell. Then we found that upregulation of miR-137 dramatically inhibited OSCC cell proliferation, colony formation, mi gration and invasion than the cells transfected with miR-137 mimic control. What is more, we made use of luciferase reporter assay and western blot proved that BRD4 as a potential target gene of miR-137 in OSCC cell. Moreover, both the qRT-PCR and western blot showed that the level of BRD4 can be negatively regulated by miR-137, which was play the role by binding with a site in the BRD4 3'-UTR. As a member of the bromodomain and extraterminal (BET), BRD4 play an important role in the pathogenesis of multiple human cancers, and it has been confirmed to be a therapeutic target for many kinds of tumors [8, 10, 32, 33].

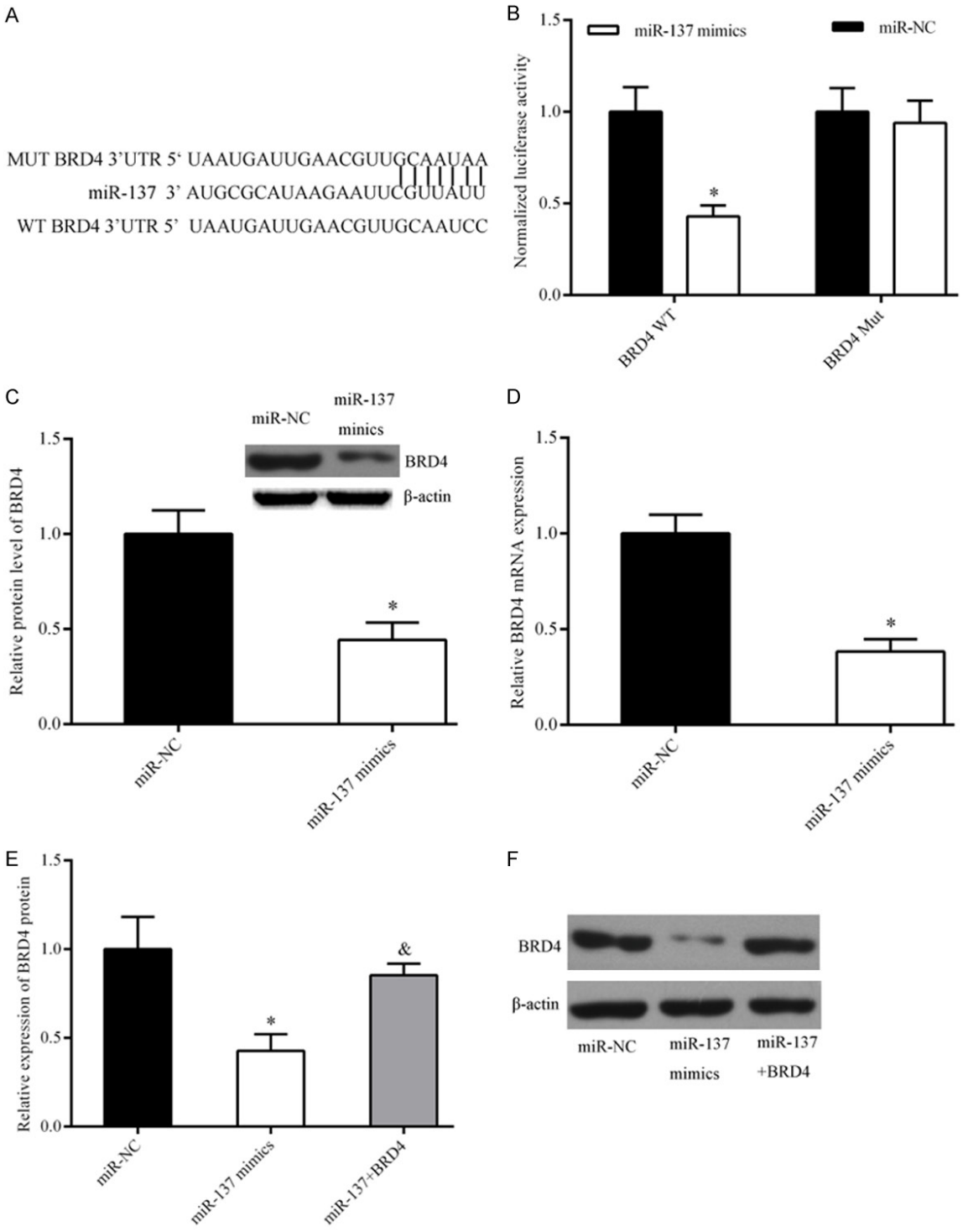

Figure 4. BRD4 is a direct target of miR-137. A: Sequence of miR-137 and the BRD4 3'-UTR, which contains a predicted miR-137 biding site. B: Luciferase assay in SCC4 cells co-transfected with miR-137 mimics and mimic control which containing the BRD4 3'-UTR (WT) or a mutant (Mut) (*P<0.05 when compared with the miR-NC). C, D: miR-137 transfection suppressed the BRD4 protein and gene levels (*P<0.05 when compared with the miR-NC). E, F: Transfection of BRD4 recovered the miR-137 induced down-regulation of BRD4 (*P<0.05 when compared with the miR-NC, &P<0.05 when compared with the miR-137 mimics).

Recent studies have proven that suppression BRD4 inhibits the growth of many kinds of cancers by restraining tumor cell viability and promoting cell apoptosis [10, 34]. In this study, we

transfected BRD4 into miR-137-overexpressing cells, results showed that overexpression of BRD4 recovery the proliferation, migration and invasion of OSCC cells suppressed by upregulation miR-137. These results illustrated that miR-137 might act as a tumor suppressor in OSCC by targeting BRD4.

To sum up, our study demonstrated that the level of miR-137 decreased in OSCC tissues and cell lines. Upregulation of miR-137 inhibited the OSCC cells proliferation, migration and invasion. At the same time, we confirmed that BRD4 was a direct target gene of miR-137. This study revealed that miR-137 may be a new therapeutic target for OSCC.

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Disclosure of conflict of interest

None.

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