miR-204 enhances p27 mRNA stability by targeting Brd4 in head and neck squamous cell carcinoma

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Abstract. The present study aimed to explore the function of microRNA (miR)-204 in modulating cyclin-dependent kinase inhibitor 1B (p27) mRNA stability in head and neck squamous cell carcinoma (HNSCC). Briefly, reverse transcription quantitative polymerase chain reaction and western blot analysis were used to detect miR-204 and Brd4 level. Cell viability, cell cycle and cell apoptosis were used to investigate the effects of miR-204. Additional luciferase reporter and mRNA stability assays were used to explore the mechanisms contributing to miR-204 effects. Here, miR-204 was downregulated in HNSCC tissues compared with the adjacent normal tissues. The expression levels of miR-204 and bromodomain-containing protein 4 (Brd4) were negatively associated in HNSCC tissues. Ectopic expression of miR-204 inhibited HNSCC cell proliferation, promoted cell cycle arrest at the G1/S phase and promoted cell apoptosis compared with control cells. Additionally, upregulation of miR-204 expression levels enhanced p27 mRNA stability. Notably, Brd4 was identified as a target of miR-204, and the co-expression of Brd4 with miR-204 mimics attenuated the inhibitory effects of miR-204 on cell proliferation and enhanced p27 mRNA stability compared with control cells. Thus, it was concluded that miR-204 functions as a tumor suppressor by enhancing p27 mRNA stability through targeting Brd4 in HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most prevalent cancer types globally (1). Although there has been extensive progress in HNSCC treatment, the overall survival rate remains at 33.24%, and numerous patients exhibit metastatic recurrence and drug resistance eventually (2). Thus, exploring novel targets and associated mechanisms that inhibit or facilitate HNSCC progression is an urgent task for ameliorating HNSCC treatment.

MicroRNAs (miRNAs/miRs) are a type of non-coding RNA (18-24 nucleotides in length) that regulate mRNA expression by binding to the 3'untranslated regions (UTR) of target mRNAs (3). Increasingly, evidence has revealed that miR-204 is downregulated in breast cancer (4), glioma (5), gastric cancer (6) and acute myeloid leukemia (7). However, to the best of our knowledge, no information is available regarding the function of miR-204 in HNSCC progression. One previous study has demonstrated that Brd4 overexpression may decrease cyclin-dependent kinase inhibitor 1B (p27) mRNA stability or transcription in neuroendocrine tumor cells (8). Additionally, the p27 protein has been demonstrated to serve a critical function in inhibiting tumor progression (9). Nevertheless, whether the Brd4/p27 regulatory route is involved in HNSCC progression has not yet been reported, to the best of our knowledge. Thus, it may be hypothesized that miR-204 enhances p27 mRNA stability and thus inhibits HNSCC development via targeting Brd4. The potential mechanism of this effect may be the involvement of the Brd4/p27 pathway.

In the present study, the function of miR-204 in modulating cyclin-dependent kinase inhibitor 1B (p27) mRNA stability in HNSCC was investigated.

Materials and methods

HNSCC clinical samples and cell culture. A total of 23 pairs of HNSCC paraffin-embedded tissue samples were randomly selected from the Affiliated Hospital of Jining Medical College (Jining, China) between October 2014 and November 2016. The thickness of these tissue samples was 4-7 μm, and were used for detecting miR-204 and Brd4 levels via reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Written informed consent from all patients and ethical approval from the Hospital Ethic Review Committee was obtained. HNSCC cell lines SCC25, Cal27, SCC4, HN12, HN13 and FaDu were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai,

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China). All cell lines were maintained in Dulbecco's modified Eagle Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO_2 at 37˚C.

Bioinformatics. TargetScan v6.2 (http://www.targetscan.org/; August 23, 2017) was used to predict miRNAs that could potentially bind to Brd4 3'UTR. In this software, gene name 'Brd4' and 'ENST00000263377.2' were used as the search terms.

miRNA, plasmids and transfection. miR-204 mimics/inhibitors and the associated negative control (NC) were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Brd4 coding sequences were cloned into the pcDNA 3.1 vector (plasmid no. 70219, Addgene, Inc., Cambridge, MA, USA) and the construct was verified using DNA sequencing, which is denoted as Brd4-CDS. Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc) was used for miR-204 mimics (50 nm), inhibitor (50 nm) and plasmid (2.5 μg) transfection. A total of 48 h after transfection, the expression of genes was detected using RT-qPCR, and the protein expression of genes was tested using western blotting. The sequences of the primers used are included in Table I.

Cell proliferation assay. FaDu cells with or without miR-204 overexpression were seeded in 96-well plates at 3,000 cells/well and incubated at 37˚C the day prior to transfection. The cell growth rate was evaluated using a Cell Counting Kit-8 assay (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol.

Cell cycle assay. Following transfection for 48 h, FaDu cells were starved using FBS-free DMEM medium for 12 h and harvested, re-suspended in 4˚C 70% ethanol and stored overnight at -20˚C. Then the cell cycle assays were performed using a cell cycle detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. The percentage of cells in each phase was analyzed using ModFit LT 4.0 software (Verity Software House, Inc., Topsham, ME, USA).

Cell apoptosis assay. Cell apoptotic rate was determined using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (Beyotime Institute of Biotechnology) with flow cytometry analysis (BD Biosciences, San Jose, CA, USA). Briefly, subsequent to transfection for 48 h, $5x10^5$ FaDu cells were harvested and washed with ice-cold PBS for one time. Then cells were double stained with Annexin V-FITC (50 nM) and PI (50 nM) for 10 min at room temperature, and washed with ice-cold PBS twice following the manufacturer's protocol. 10,000 cells were collected and FlowJo v10 software (FlowJo LLC, Ashland, OR, USA) was used to analyze the data, which were expressed as cell percentage.

Luciferase reporter assays. For miRNA target validation, pMIR-Report vector (cat. no. AM5795; Thermo Fisher Scientific, Inc.) was used to introduce the fragment of Brd4 3'UTR containing the wild-type (WT) or the mutant (MUT) binding sites for miRNA-204, referred to as Luc-Brd4-WT and Luc-Brd4-MUT, respectively. A total of $1x10^6$ FaDu cells were co-transfected with Luc-Brd4-WT or Luc-Brd4-MUT and miR-204 mimics or NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 48 h later, cells were lysed with Reporter lysis buffer (cat. no. E397A; Promega Corporation, Madison, WI, USA) and luciferase activity was measured with VivoGlo Luciferin kit (cat. no. P1041; Promega Corporation) using a luminometer (Thermo Fisher Scientific, Inc.). β-gal was utilized to normalize the transfection efficiency.

RT-qPCR. Total RNA was extracted from FaDu cells using TRizol® reagent (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The first strand cDNA was synthesized using M-MLV reverse transcriptase (Promega Corporation, Madison, WI, USA) and using an oligo(dT) 18 primer (5'-TTTTTTTTTTTTTTTTT-3'). The thermocycler conditions were 30˚C for 10 min, 42˚C for 1 h and 95˚C for 10 min. RT-qPCR was performed on an ABI Prism 7500 Sequence Detector (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the SYBR-Green RT-qPCR kit (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The thermocycler conditions were 94˚C for 5 min, followed by 94˚C for 40 sec, 60˚C for 30 sec and 72˚C for 1 min, for 40 cycles, and then 72˚C for 10 min. For the analysis of mRNA expression, all primers used are included in Table I. GAPDH served as a reference gene. For miRNA expression detection, the miR-204 primer and U6 primer were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). U6 was used as an internal reference gene. Relative quantification was calculated using the $2^{-\Delta\Delta Cq}$ method (10).

Western blotting. FaDu cells were lysed using RIPA buffer (cat. no. P0013B, Beyotime Institute of Biotechnology) and the concentration of protein was determined utilizing a BCA Protein Assay kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). A total of 30 μg protein was separated using 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk at room temperature for 1 h, and then incubated with primary antibodies against Brd4 (dilution 1:1,000; cat. no. ab75898), p27 (dilution 1:5,000; cat. no. ab32034) and β-actin (dilution 1:10,000; cat. no. ab8226) at 4˚C overnight, which were purchased from Abcam (Cambridge, MA, USA). Following washing three times with tris-buffered saline with 0.5% Tween-20, the membranes were incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit (cat. no. A0208) and goat anti-mouse antibody (cat. no. A0216) (both Beyotime Institute of Biotechnology; and both dilution, 1:5,000) at room temperature for 1 h. The membranes were subsequently developed using an ECL system (Thermo Fisher Scientific, Inc.). β-actin was used as an internal control.

mRNA stability assays. FaDu cells were performed on this experiment. miR-204 was induced by transfection with miR-204 mimics for 48 h. Then, *de novo* RNA synthesis was blocked at 37˚C for 2, 4 and 6 h by the addition of 5 μg/ml of actinomycin D (ActD; Apexbio, Houston, TX, USA) into DMEM. Total RNA was harvested at the 2, 4 and 6 h, and mRNA expression was detected using RT-qPCR as

Table I. Primer sequences.

Gene	Sequence (5' to 3')
Brd4-CDS forward (SpeI)	CCCAAGCTTATGTCTGCGGAGAGCGGCCCTGGGA
Brd4-CDS reverse (XbaI)	CGCGGATCCTCAGAAAAGATTTTCTTCAAATATT
GAPDH forward	CGGAGTCAACGGATTTGGTCGTAT
GAPDH reverse	AGCCTTCTCCATGGTGGTGAAGAC
Brd4-RT-qPCR forward	CAGTGACAGTTCGACTGATGACTC
Brd4-RT-qPCR reverse	TTTCCTTTTGTGCTTTTCTTTTTT
p27-RT-qPCR forward	CTCCAAGACAAACAGCGGAAAATC
p27-RT-qPCR reverse	GTCTGTGTGCAGGCCATGACATCT

Brd4, bromodomain-containing protein 4; CDS, coding sequences; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; p27, cyclin-dependent kinase inhibitor 1B.

aforementioned. The mRNA half-life was determined by comparing to the mRNA levels prior to the addition of ActD.

Statistical analysis. GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA) was used to analyze the data. All data were obtained from at least three independent experiments ($n \geq 3$) and presented as the mean ± standard deviation. Datasets with only two groups were analyzed using an unpaired student's t-test. Differences between multiple groups were analyzed using one-way analysis of variance followed by a Tukey-Kramer post-hoc test, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-204 is downregulated and negatively associated with Brd4 expression levels in HNSCC tumor samples. RT-qPCR was performed to detect the expression levels of miR-204 in HNSCC tumor and adjacent normal tissues. The levels of miR-204 were significantly downregulated in HNSCC tumor types compared with the normal adjacent tissues ($P=0.0016$; Fig. 1A). In contrast, Brd4 mRNA expression level was significantly higher in HNSCC tumor types compared with normal tissues ($P<0.01$; Fig. 1B). Interestingly, the miR-204 expression level was significantly negatively associated with Brd4 expression in HNSCC tissues ($P<0.01$; Fig. 1C). These results indicate that miR-204 may hold a suppressive function in HNSCC tumor types, which is associated with Brd4 expression.

Effects of miR-204 on HNSCC cell proliferation, cycle and apoptosis. Initially, the expression levels of miR-204 in seven HNSCC cell lines (SCC25, Cal27, SCC4, HN12, HN13 and FaDu) were examined, and the lowest level of miR-204, compared with all other cell lines, was observed in FaDu cells (Fig. 2A). Then, miR-204 expression levels were upregulated using miR-204 mimics transfection in FaDu cells. Transfection with miR-204 mimics significantly upregulated miR-204 level in FaDu cells compared with the control ($P<0.01$; Fig. 2B). A cell proliferation assay revealed that the upregulation of miR-204 expression levels significantly inhibited cell proliferation in HNSCC cells compared with the control ($P<0.01$; Fig. 2C). Additionally, a cell cycle assay indicated that the ectopic expression of miR-204 promoted cell cycle arrest in G1/S phase compared with the control (Fig. 2D). Additionally, miR-204 overexpression was demonstrated to enhance cell apoptosis in HNSCC cells compared with the control (Fig. 2E).

Brd4 is potential target of miR-204. TargetScan (version 6.2, http://www.targetscan.org/) was used to predict potential targets of miR-204 and it was revealed that Brd4 may be a potential target with two putative miR-204 binding sites within its 3'UTR (Fig. 3A). Brd4 expression was measured in SCC25, Cal27, SCC4, HN12, HN13 and FaDu cells via RT-qPCR and western blotting. It was revealed that there were higher levels of mRNA expression of Brd4 in FaDu cells compared with in any other cells (Fig. 3B and C). Fig. 3D revealed that the co-transfection of FaDu cells with miR-204 mimics and the Luc-Brd4-WT constructs resulted in a significant reduction in luciferase activity compared with transfection with NC ($P<0.05$). Conversely, no significant effect on the luciferase activity of Luc-Brd4-MUT was observed following miR-204 upregulation, suggesting that Brd4 is a direct target of miR-204. RT-qPCR experiments further confirmed that upregulation of miR-204 significantly decreased Brd4 mRNA expression levels in HNSCC cells ($P<0.01$; Fig. 3E), and western blotting experiments produced similar results (Fig. 3F). In summary, it was confirmed that Brd4 is a potential target of miR-204 in FaDu cells.

miR-204 enhances p27 mRNA stability via targeting Brd4. It was further examined whether miR-204 may affect the expression of anti-proliferative factors including p27. As expected, transfection with miR-204 mimics significantly increased p27 mRNA expression levels ($P<0.01$; Fig. 4A) and protein expression in FaDu cells (Fig. 4B), which indicates that miR-204 modulates p27 expression at the transcriptional level. To determine whether miR-204 influences p27 mRNA stability, HNSCC cells were transfected with miR-204 for 48 h, followed by ActD treatment. Fig. 4C demonstrated that transfection with miR-204 mimics delayed the reduction of p27 mRNA. As Brd4 may bind to the acetylated histones in the chromatin, resulting in the suppression of gene transcription (11), it was hypothesized that miR-204 enhances p27 mRNA stability by targeting Brd4. When FaDu cells were co-transfected with miR-204 mimics with Brd4 overexpression plasmid (Brd4-CDS), Brd4 overexpression attenuated or even reversed the promotive effect of miR-204 on p27 mRNA stability and expression (Fig. 4D and E). Overall, the results of the present study reveal that miR-204 may enhance p27 mRNA stability through the use of Brd4.

miR-204 exerts its effects on HNSCC cell proliferation, cycle and apoptosis partially through Brd4. Further investigations were conducted into whether the functional effects of miR-204

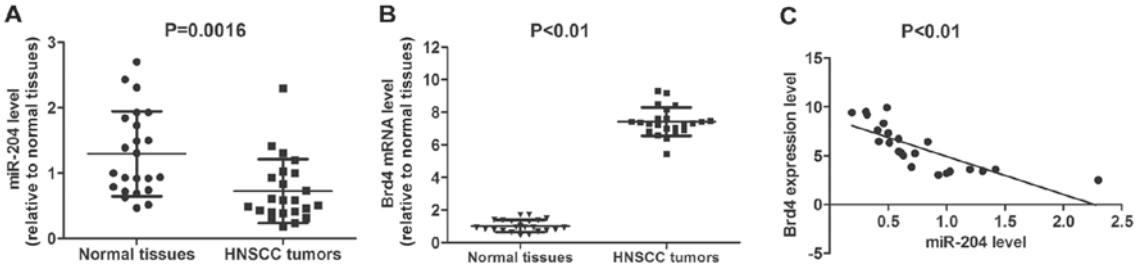

Figure 1. miR-204 is downregulated and negatively associated with Brd4 expression in HNSCC tumor samples. (A) miR-204 expression level was examined in HNSCC and adjacent normal tissues via RT-qPCR analysis, and a significant difference was observed. (B) mRNA expression level of Brd4 was detected in HNSCC and adjacent normal tissues via RT-qPCR, and a significant difference was observed. (C) Expression levels of miR-204 and Brd4 in HNSCC tissues demonstrated a significant negative association with one another. Data were presented as the mean ± standard deviation. miR, microRNA; Brd4, bromodomain-containing protein 4; HNSCC, head and neck squamous cell carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

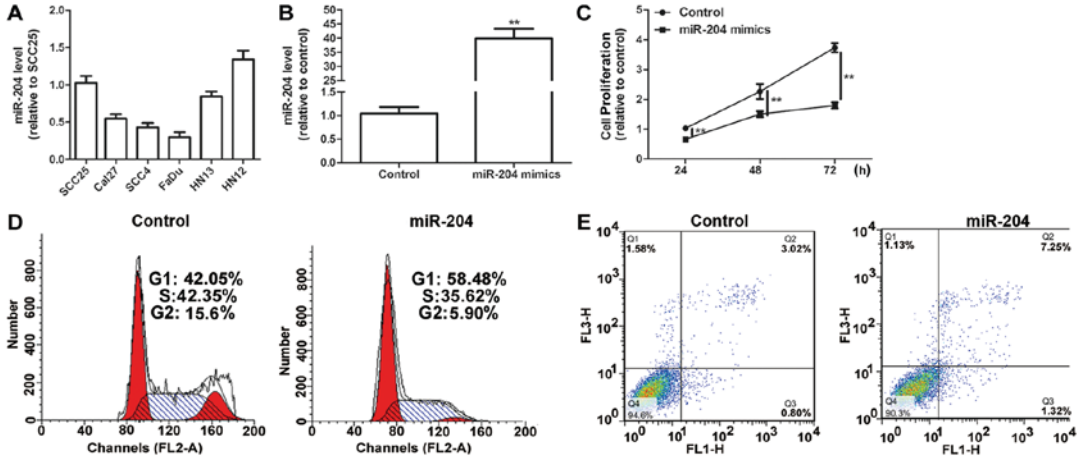

Figure 2. Effects of miR-204 on HNSCC cell proliferation, cycle and apoptosis. (A) miR-204 expression levels were detected in various HNSCC cell lines using RT-qPCR. (B) Transfection efficiency of miR-204 mimics was determined in FaDu cells via an RT-qPCR assay. **P<0.01 vs. control. (C) Cell proliferation of FaDu cells transfected with miR-204 mimics or a control was evaluated using a Cell Counting Kit-8 assay. **P<0.01 with comparisons shown by lines. (D) Changes in the cell cycle were detected in FaDu cells transfected with miR-204 mimics or a control. (E) Apoptosis was examined in FaDu cells transfected with miR-204 mimics or a control. Data were presented as the mean ± standard deviation. miR, microRNA; HNSCC, head and neck squamous cell carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

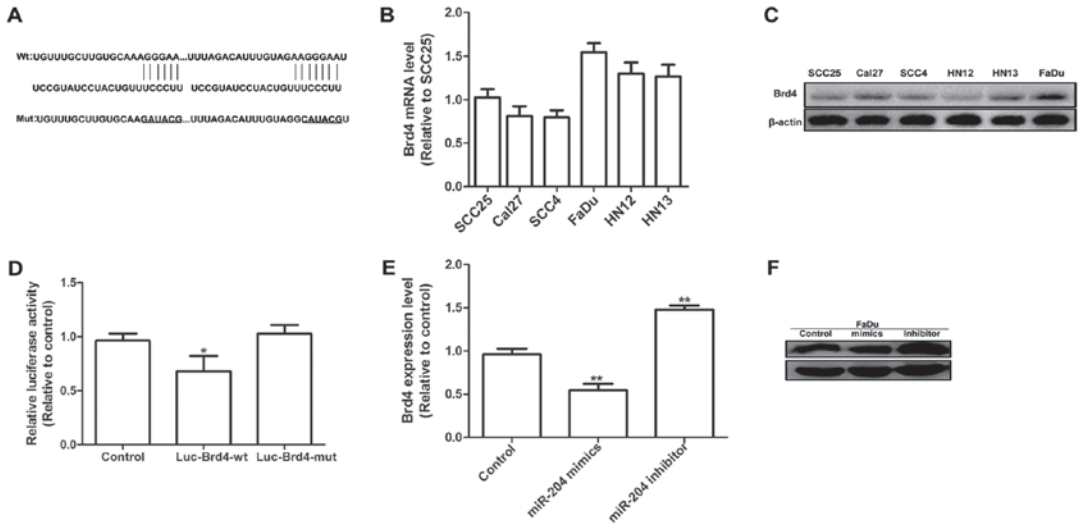

Figure 3. Brd4 is potential target of miR-204. (A) Predicted binding sites for miR-204 in the 3'-untranslated region of human Brd4 mRNA (wild type and mutant). Brd4 mRNA expression levels and protein expression were detected in various head and neck squamous cell carcinoma cell lines (SCC25, Cal27, SCC4, HN12, HN13 and FaDu) by (B) RT-qPCR and (C) western blotting. (D) FaDu cells were co-transfected with Luc-Brd4-WT or Luc-Brd4-MUT, miR-204 mimics and β-gal control plasmid for 48 h, then luciferase activity was measured and normalized to β-gal activity. *P<0.05 vs. control. (E) RT-qPCR and (F) western blotting results of Brd4 expression in FaDu cells following transfection with miR-204 mimics for 48 h. Data were presented as the mean ± standard deviation. **P<0.01 vs. control. miR, microRNA; Brd4, bromodomain-containing protein 4; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; Luc, luciferase; WT, wild type; MUT, mutant.

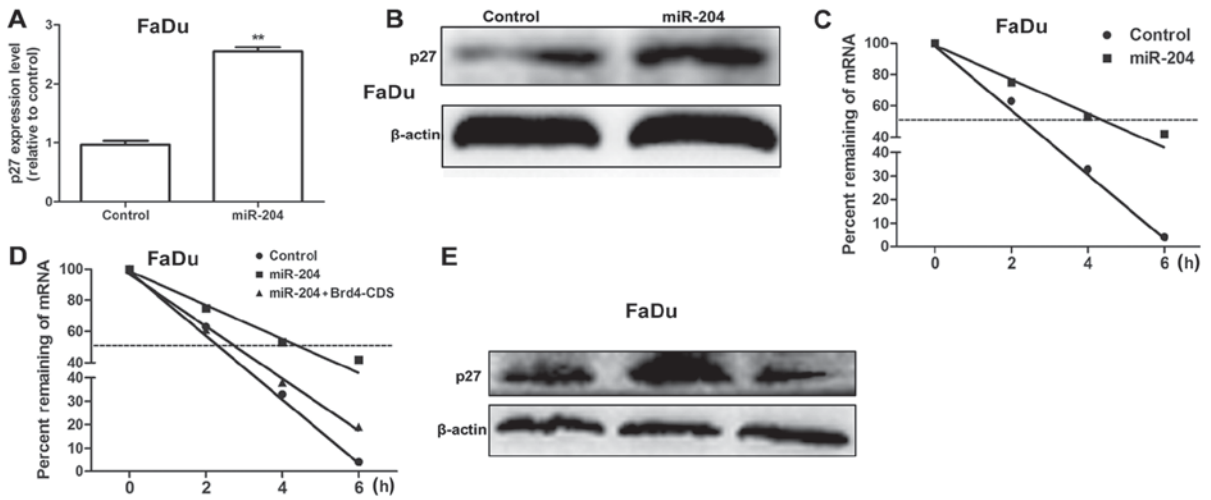

Figure 4. miR-204 enhances p27 mRNA stability via targeting Brd4. (A) mRNA expression levels and (B) protein expression of p27 were examined in FaDu cells with miR-204 transfection or control. (C) FaDu cells with miR-204 mimics transfection were treated with ActD (5 μg/ml) for the indicated times. p27 mRNA levels were measured using RT-qPCR and the percentage of mRNA was plotted. (D) FaDu cells with miR-204 mimics transfection plus Brd4-CDS or not were treated with ActD (2.5 μg/ml) for the indicated times. p27 mRNA expression levels were measured using RT-qPCR and the percentage of mRNA that remained was plotted. (E) p27 protein expression was detected in FaDu cells. Data were presented as the mean ± standard deviation. **P<0.01 vs. control. miR, microRNA; p27, cyclin-dependent kinase inhibitor 1B; Brd4, bromodomain-containing protein 4; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ActD, actinomycin D; CDS, construct verified by DNA sequencing.

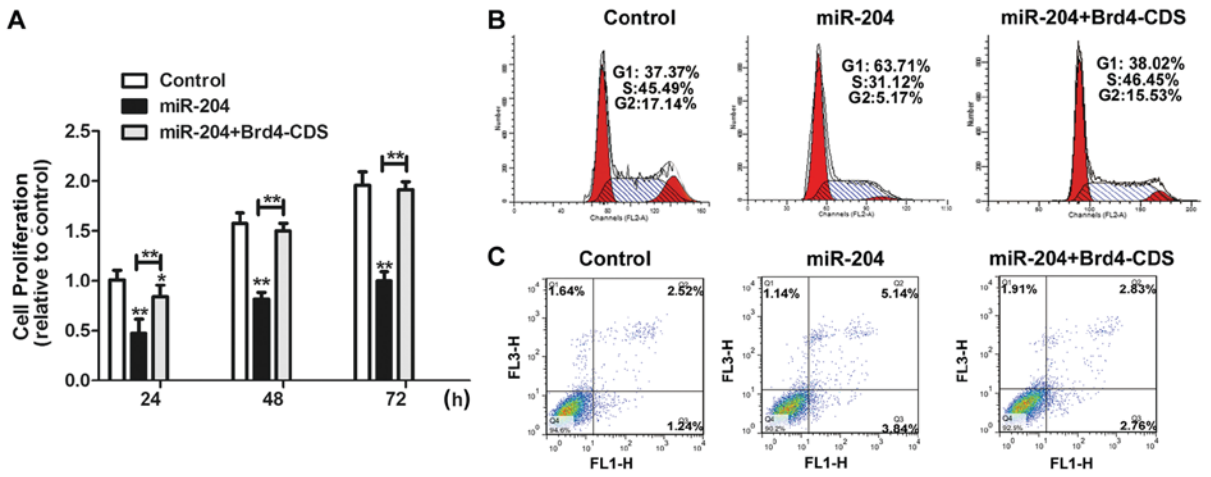

Figure 5. miR-204 exerts its effects on HNSCC cell proliferation, cycle and apoptosis partially through Brd4. (A) FaDu cells were transfected with miR-204 mimics plus Brd4-CDS or alone, then the cell proliferation was examined using a Cell Counting Kit-8 assay. (B) Cell cycle and (C) apoptosis were evaluated in FaDu cells were transfected with miR-204 mimics plus Brd4-CDS or alone. Data were presented as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. Control. miR, microRNA; HNSCC, head and neck squamous cell carcinoma; Brd4, bromodomain-containing protein 4; CDS, construct verified by DNA sequencing.

are dependent on Brd4 expression. Brd4-CDS was co-transfected with miR-204 mimics in FaDu cells. As presented in Fig. 5A and B, overexpression of Brd4 significantly counteracted the inhibitory effects of miR-204 on cell proliferation (P<0.05) and counteracted the inhibitory effects of miR-204 on the percentage of cells in the G1/S phase. Furthermore, the promotive effects of miR-204 on cell apoptosis were decreased by Brd4 overexpression (Fig. 5C). Therefore, these results demonstrate that the inhibitory effects that miR-204 exerts on HNSCC progression is partially dependent on Brd4 expression.

Discussion

miR-204 is known as a multifunctional miRNA involved in tumor development (12-14). However, to the best of our knowledge, the functions of miR-204 in HNSCC have never been reported.

The present study focuses on the functions of miR-204 on HNSCC cell proliferation and apoptosis. The clinical tissue samples revealed that miR-204 was downregulated, however Brd4 expression was upregulated in HNSCC tumor tissues, and their expression were negatively associated. The targeted association between miR-204 and Brd4 was confirmed using a luciferase reporter assay, RT-qPCR and western blotting. As Brd4 may decrease p27 mRNA stability or transcription in neuroendocrine tumor cells (8), it was further investigated whether p27 participates in HNSCC progression regulated by miR-204 and the results of the present study revealed that the Brd4/p27 pathway regulated by miR-204 serves an essential role in HNSCC progression.

To the best of our knowledge, the present study is the first to demonstrate the function of miR-204 in HNSCC progression. However, as miR-204 may exert inhibitory effects on tumor angiogenesis and metastasis (4,15), it is additionally interesting to note whether miR-204 exerts inhibitory functions on HNSCC metastasis and angiogenesis. Notably, Brd4 was initially identified as a potential target of miR-204 and was involved in the p27 mRNA stability regulated by miR-204 in HNSCC. Future experiments should focus on whether the miR-204/Brd4 regulatory route exists in other tumor types. Although the Brd4 inhibitor IBET has been demonstrated to exert inhibitory functions in the development of various tumor types (8,16,17), exploring the functions of miR-204 may provide another clue to develop novel Brd4 inhibitors. In summary, the results of the present study offer novel insight into the function of miR-204 in HNSCC progression, suggesting use as a compelling biomarker or therapeutic target for HNSCC progression.

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Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

Authors' contributions

CW and YZ designed the study. YZ and GC analyzed the data. CW, YZ, DZ and YW performed the experiments. CW wrote the manuscript.

Ethics approval and consent to participate

Written informed consent from all patients and ethical approval from was obtained the Hospital Ethic Review Committee.

Patient consent for publication

The manuscript declared that the patients have provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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