MiRNA-516b inhibits ameloblastoma cell proliferation and invasion by regulating MYCBP/c-myc/RECK/MMP pathway

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Background: Ameloblastoma (AM) is a frequent odontogenic tumor, which is associated with high risk of recurrence. Previous studies have reported dysregulation of miRNA-516b (miR-516b is dysregulated in some malignancies); however, its biological function in AM is poorly understood.

Methods: Quantitative real-time PCR was used to estimate miR-516b expression in AM tissues and cell line. CCK8, flow cytometry, wound scratch, and Transwell invasion assays were used to determine cell proliferation, cell cycle and apoptosis, migration, and invasion in AM cells, respectively. Using bioinformatics analyses and luciferase assay, a new target of miR-516b was identified, and Western blot was used to detect downstream effector proteins of miR-516b.

Results: miR-516b was found to be markedly downregulated in AM tissues as compared to normal tissues. Enforced expression of miR-516b significantly inhibited cell proliferation, migration, invasion, and induced cell cycle G0/G1 phase arrest and apoptosis in AM cells. The oncogene, MYCBP, was identified as a potential miR-516b target and its depletion by siRNAs has similar effects as miR-516b overexpression on cell proliferation and mobility. However, rescued MYCBP expression partially reversed the inhibitory effects of miR-516b in AM cell lines. Further, c-myc, RECK, MMP2, and MMP9 were identified as downstream effector proteins of miR-516b.

Conclusions: These results suggested that the potential suppressive role of miR-516bin AM was due to the suppression of MYCBP/c-myc/RECK/MMP pathway.

Keywords: Ameloblastoma (AM); miR-516b; cell proliferation; invasion; MYCBP/c-myc/RECK/MMP pathway

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Introduction

Ameloblastoma (AM) is a frequent odontogenic tumor originating from dental epithelium. AM is characterized as invasively aggressive owing to its local invasiveness ability and higher risk of recurrence (1,2). AM accounts for more than 30% of all odontogenic tumors (3), which are typically classified as multicystic, peripheral, desmoplastic, unicystic, and ameloblastic carcinoma based on the World Health Organization classification of head and neck tumors (4). While surgical treatments are effective in preventing AM, severe facial deformity and psychological trauma are some of the consequences associated with the use of surgical treatments (5). Thus, identification of molecular aberrations is critical in order to find effective therapeutic strategies for the treatment of AM.

MicroRNAs (miRs) are small, non-coding endogenous
RNAs (19-22 nucleotides) derived from double-strand RNA (6). miRs play important regulatory function by suppressing target gene expression by binding to the 3’ untranslated region (UTR) of target mRNA, thereby resulting in the degradation of target mRNA or inhibiting translation (7). Many miRs are highly evolutionarily conserved among distant species, thus playing an important role in essential biological processes, such as cell proliferation, apoptosis, migration and signal transduction (6,8,9). Increasing evidence has showed that aberrant miRNAs expression exhibits oncogenic or tumor suppressive roles in human cancers (10-12). Diniz et al. has demonstrated that lower miR-15a/16-1 expression promotes the development of odontogenic tumor, thereby indicating an etiological and therapeutic role of miRs in odontogenic tumor (13). Recently, miR-516b has been reported to be significantly downregulated in several malignant tumors, including non-small cell lung cancer (NSCLC)(14), primary melanoma (15), and prolactinomas (16), thus suggesting its role in tumor suppression. However, to our knowledge, no studies have evaluated the expression and biological function of miR-516b in AM.

Thus, we pay an attention on the critical role of miR-516b in AM. We determined the expression levels of miR-516bin AM samples and normal cells. In this study, we investigated the role of miR-516b in AM and identified the underlying molecular mechanisms.

**Methods**

**Tissue collection**

Fresh AM tissues were obtained from AM patients, who received surgical treatment without any pre-operative chemotherapy or radiotherapy, at Sun Yat-sen Memorial Hospital, China. Normal oral mucosa was used as control. These tissues were frozen in liquid nitrogen and stored at −80 °C for the subsequent experiments. The study and the samples used were approved by the ethics committee of Sun Yat-sen Memorial Hospital. Written informed consent was obtained from all the patients before administering the clinical materials.

**Cell culture and transfection**

The human immortalized AM cell line, hTERT-AM and normal epithelial cells were obtained from Guanghua School and Hospital of Stomatological Research, Sun Yat-sen University (Guangzhou, China). The samples were cultured in α-MEM supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA) and 200 μg/mL G418 (Gibco, Grand Island, NY, USA) in a humidified atmosphere containing 5% CO₂ at 37 °C.

For cell transfection, the cells were cultured in a complete medium without antibiotics for at least 24 hours. The cells were washed with 1× PBS (pH 7.4) and transiently transfected with miR-516b mimic, mimic negative control (NC), MYCBP siRNA, and scramble siRNA (GenePharma, Shanghai, China) using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. For MYCBP overexpression, the cells transfected with miR-516b mimic were plated in 24-well plates, pcDNA3.0-MYCBP and pcDNA3.0 blank vector (Invitrogen, USA) were transfected into the cultured cells, respectively, with Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer’s instructions.

**RNA isolation and quantitative real time PCR (qRT-PCR)**

Total RNA from tissues and cells was isolated using RNAiso (TaKaRa, Dalian, China) and reverse transcribed using PrimeScript RT reagent kit(TaKaRa) according to the manufacturer’s instructions. The mRNA expression levels were detected using the SYBR-Green PCR Master Mix kit (Takara) in PCR reaction. Reaction steps were as follows: 95 °C for 30 s, as the first step in a loop; 95 °C for 5 s, 60 °C for 34 s, as the second step, for 40 cycles. Then qRT-PCR was carried out using an ABI PRISM 7900HT Sequence Detection System (Thermo Fisher Scientific, Inc., MA, USA). The relative expression values of miR-516b was calculated and normalized to endogenous small nuclear RNA U6 control in each sample using the 2^−ΔΔCT method. The primer sequences used in this study are as follows:

- Human miR-516b;
- RT: CTCAACTGGTGTCGTGGAGTCGGAAATTTCAGTTGAGAAAGTGCT;
- miR-516b: F: ACACTCCAGCTGGGATCTGGA GGTAAGAAGC;
- U6 F: CTCGCTTCGGCAGCACA;
- U6 R: AACGCTTCACGAATTTGCGT. Each sample was run in triplicates.

**Cellular viability**

After plating cells in 96-well microtiter plates (Corning
Costar, Corning, NY, USA) at 1.0×10^3/well, cellular viability was determined after 24, 48 and 72 h using CCK-8 test (Dojindo, Kumamoto, Japan) by determining the absorbance of the converted dye at 450 nm.

**Cell cycle and apoptosis analyses**

Following cell transfection, the AM cells were seeded at a density of 3×10^5 cells per well in 6-cm dishes and collected until 80% confluence. For cell cycle analysis, the supernatant was discarded after centrifugation, and attached cells were harvested. The cells were fixed in cold 70% ethanol for 30 min at 4 °C and stained with propidium iodide (PI) solution (Sigma-Aldrich China, Inc., Beijing, China). Histograms of cell cycle distribution in G0/G, S, and G2/M phases were analyzed using a FACSort flow cytometer (Becton Dickinson, CA, USA) according to the manufacturer’s instructions. For apoptosis analysis, the cells were stained with Annexin V-APC/7-AAD kit (BestBio, Shanghai, China). Stained cells were analyzed using FACSort flow cytometer (Becton Dickinson, CA).

**Wound scratch assay**

At 48 h post-transfection, the cells were incubated overnight until they had reached 85–95% confluence. Three scratches/well were made in each plate, using a 200 μL plastic filter tip. The detached cells were washed off using serum-free medium. The lodged cells were incubated in fresh complete medium at 37 °C in a 5% CO₂ atmosphere. At 24 h post-scratching, the wound areas were photographed and observed using a microscope. The quantification of wound closure was performed using Zen2012 software by measuring the percentage of the wound area as compared to the total cell covered area. All scratch assays were performed in triplicates and the experiment was repeated thrice.

**In vitro transwell invasion assay**

The Transwell invasion assay was used assess the invasive ability of AM cells under different treatments. Briefly, the cells were cultured in serum-free media in the upper Transwell culture chambers, with 8-μm pore filter inserts pre-coated with BD Matrigel™ Basement Membrane Matrix (BD Biosciences, San Jose, CA, USA) overnight. The medium with 10% FBS was added to the lower chamber as chemoattractant. After 24 h of incubation, the cells attaching to the lower surface were fixed with 100% methanol, and stained with 0.1% crystal violet. The number of invasive cells on the lower chamber was counted in high-power fields (magnification, ×200), and the average invasive number was calculated as cell counts. All invasion assays were performed in triplicates and each independent experiment was repeated thrice.

**Luciferase reporter assays**

A dual luciferase assay was performed to confirm whether MYCBP directly bind to miR-516b. Briefly, the AM cells were reseeded in 24-well plates for 24 h and were transiently co-transfected with 0.3 μg MYCBP-3’-UTR-wild type or MYCBP-3’-UTR-mutant reporter plasmid (Agilent Technologies, USA), followed by transfection with corresponding 50 nM miR-51b mimic or miR-control using Lipofectamine 2000 (Invitrogen). After 48 h, Firefly and Renilla luciferase activities were analyzed using the Dual Luciferase Assay (Promega, Madison, WI, USA) according to the manufacturer’s protocol. Each test was repeated thrice and each independent experiment was performed in triplicates.

**Western blot analysis**

Fresh AM tissue samples and cells were homogenized and lysed in a buffer containing 1X protease inhibitor cocktail (Thermo Fisher Scientific, Inc.). The protein concentration was determined using Bradford assay (Bio-Rad Laboratories, USA). Equivalent quantities of protein (30 μg) were separated on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (10%) and transferred to a PVDF membrane. The membrane was blocked with 10% defatted milk and incubated overnight with primary antibodies targeting c-mycbp (Abcam, ab139534, 1:1000), c-myc (Abcam, ab10910, 1:000), RECK (Abcam, ab88249, 1:000), MMP2 (Abcam, ab14311, 1:000), MMP9 (Abcam, ab73734, 1:000), and GAPDH (Abcam, ab88249, 1:000), which are purchased from ABCAM (Cambridge, England). After washing with PBST thrice, the membrane was incubated with the corresponding secondary antibodies. The bound antibodies were detected using the enhanced chemiluminescence kit (Santa Cruz, USA).

**Statistical analysis**
Results

Regulation of MiR-516b in AM tissue specimens and cells

The expression levels of miR-516b were determined in all AM specimens and patient-matched normal specimens. The result showed that the expression of miR-516b was significantly downregulated in AM tissues as compared to the adjacent normal tissues (Figure 1A and Table 1, P<0.01). Additionally, the expression of miR-516b in hTERT-AM cell line was decreased as compared to the cells infected with sh-c-myc (Figure 1B and Table 2, P<0.001). These findings indicated that miR-516b plays an important role in the development of AM.

Effect of miR-516b on cell proliferation, cell migration and invasion, and cell cycle in AM

To investigate the biological effects of miR-516b in AM cells, the hTERT-AM cell line was first transfected with miR-516b mimic and mimic NC. Subsequently, a series of functional assays, including CCK-8, flow cytometry assay and Transwell assay, were used to identify AM cells transfected with miR-516b mimics. The CCK-8 assay showed that transfection with miR-516b mimic inhibited cell proliferation as compared to transfection with mimic NC (Figure 2A, P<0.05). Cell cycle analysis showed that the experimental group transfected with the miR-516b mimics displayed a significantly higher percentage of cells in the G0/G1 phase (68.55%±1.17% vs. 56.44%±0.97%) and a lower percentage of cells in the S phase (14.99%±0.76% vs. 25.91%±1.23%) as compared to the NC group (Figure 2B, P<0.001). Results from flow cytometry assay showed that miR-516b mimic induced early apoptosis and late apoptosis in AM cells, as indicated by increased cell percentage of early (Annexin V+/PI−) and late apoptosis (Annexin V+/PI+) as compared to NC group (Figure 2C, P<0.05, P<0.001). In addition, Wound scratch assay showed that wound areas in miR-516b mimic group was significantly

Table 1 Analysis of miR-516b expression levels in 4 paired AM tissues (T) and adjacent normal tissues (N) by quantitative real time PCR (qRT-PCR)

<table>
<thead>
<tr>
<th>Patients</th>
<th>AM tissues (T)</th>
<th>Adjacent normal tissues (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO. 1</td>
<td>1.01±0.055</td>
<td>6.65±0.068</td>
</tr>
<tr>
<td>NO. 2</td>
<td>1.31±0.044</td>
<td>3.31±0.13</td>
</tr>
<tr>
<td>NO. 3</td>
<td>0.79±0.043</td>
<td>4.47±0.050</td>
</tr>
<tr>
<td>NO. 4</td>
<td>0.65±0.058</td>
<td>1.54±0.060</td>
</tr>
</tbody>
</table>

AM, ameloblastoma.

Table 2 Analysis of miR-516b expression levels in hTERT-AM cells and cells infected with sh-c-myc by quantitative real time PCR (qRT-PCR)

<table>
<thead>
<tr>
<th>Cell</th>
<th>miR-516b expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTERT-AM cells</td>
<td>3.51±0.04</td>
</tr>
<tr>
<td>Normal cells</td>
<td>1.00±0.03</td>
</tr>
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</table>

AM, ameloblastoma.

Quantitative data is presented as the mean ± standard (SD) from three independent experiments. Significant differences in two independent groups were evaluated by t-test using SPSS 19.0 software. A value of P<0.05 was considered to be statistically significant.
decreased as compared to NC group, thereby suggesting impaired cell migration capacity induced by miR-516b overexpression (Figure 2D, P<0.05). Moreover, miR-516b was involved in the invasion of AM cells. As shown in Figure 2E, overexpression of miR-516b significantly decreased the invasion capacities of AM cells (P<0.01). Together, these results demonstrated that miR-516b suppresses tumor by suppressing proliferation and invasion in AM cells.

**Role of MYCBP in AM cells**

We identified MYCBP as a possible direct target gene of miR-516b, which is involved in the proliferation and migration of hTERT-AM cells, using predictive tools (TargetScan Human; http://www.targetscan.org/vert_71/) (Figure 3A). To verify the prediction, luciferase reporter assays were performed as described. The results showed that wild-type miR-516b significantly inhibited luciferase activity of MYCBP 3′UTR (Figure 3B, P<0.001), thereby indicting the direct regulation of miR-516b in the 3′UTR of MYCBP mRNA. Furthermore, we determined the expression of MYCBP in AM tissues and cells using Western blot analysis. As shown in Figure 3C and 3D, the expression of c-mycbp protein levels was significantly increased in AM tissues and cells as compared to control (P<0.05 or P<0.01). These results suggested that MYCBP could be an oncogene in AM, which is a direct target gene of miR-516b.

**Effect of MYCBP on proliferation, migration and invasion, and cell cycle in AM cells**

siMYCBP or scramble siRNA was transfected into hTERT-AM cells to investigate the role of MYCBP in AM cells by performing loss-of-functional experiments. As shown in Figure 4A, siMYCBP transfection significantly suppressed cell proliferation in AM cells as compared to scramble siRNA (P<0.01). Moreover, flow cytometry analysis showed that knockdown of MYCBP remarkably induced cell cycle arrest at G0/G1 phase (Figure 4B, P<0.001) and promoted early and late cell apoptosis (Figure 4C, P<0.001) in hTERT-AM cells. Similarly, it was also observed that both migration and invasion ability were significantly suppressed in siMYCBP group as compared to NC group in hTERT-AM cells, as demonstrated by Wound scratch assay (Figure 4D, P<0.05) and Transwell assay (Figure 4E, P<0.001), respectively. These findings indicated that downregulation of MYCBP could suppress cell proliferation and invasion of AM cells.

**Effect of overexpression of MYCBP on AM cells transfected with miR-516b mimics in vitro**

We determined whether upregulation of MYCBP could reverse the effects of miR-516b on the apoptosis, migration, and invasion of hTERT-AM cells. Forty-eight hours after pcDNA/MYCBP or pcDNA blank vector was transfected into miR-516b mimic, results showed that upregulation of MYCBP significantly weakened the effects of miR-516b overexpression on the G0/G phase arrest (Figure 5A) and induced early apoptosis (Figure 5B, P<0.05, P<0.001) of hTERT-AM cells. Additionally, Wound scratch assay (Figure 5C, P<0.001) and Transwell assay (Figure 5D, P<0.05, P<0.001) showed that overexpression of MYCBP could partially reverse the inhibition effects of miR-516b overexpression on cell migration and invasion in hTERT-AM cells.

**Regulation of c-myc/RECK/MMPs signaling pathway by MiR-516b**

Protein levels of c-mycbp were determined. The level of miR-516b-overexpression in AM cells was decreased as compared to control cells in a manner that was similar to c-mycbp expression induced by siMYCBP (Figure 6). Conversely, the protein levels of c-mycbp were dramatically increased when pcDNA/MYCBP was introduced into AM cells, which expressed miR-516b at a higher level (Figure 6, *, P<0.05 vs. NC group, #, P<0.05 vs. miR-516 mimics group). These results suggested that MYCBP could be a direct target and an important functional mediator of miR-516b. Further, we analyzed the expression of c-myc, RECK, and MMPs in AM cells. Similarly, miR-516b had a significant inhibitory effect on the expression of c-myc, MMP2, and MMP9, but significantly increased RECK expression in a way similar to siRNA-mediated knockdown of MYCBP (Figure 6, *, P<0.05 vs. NC group, #, P<0.05 vs. miR-516 mimics group). In addition, these effects were significantly rescued by MYCBP overexpression (Figure 6).

**Discussion**

Prognosis of AM in AM patients is poor due to its aggressive local invasiveness ability and high rate of recurrence (3). The molecular mechanisms involved in the development of AM are still not fully understood. MiRNAs have been reported to play a critical role...
Figure 2 Effects of miR-516b overexpression on proliferation, cell cycle, apoptosis, migration, and invasion of ameloblastoma (AM) cells. (A) CCK-8 assay was used to evaluate cell proliferation in hTERT-AM cells after miR-516b mimics or NC transfection; (B) representative cell cycle profile and quantification of hTERT-AM cells detected using flow cytometry with PI staining; (C) apoptotic status after transfection, as detected by flow cytometry with Annexin-FITC/PI double staining; (D) images and quantification of the migration distances in hTERT-AM cells after miR-516b mimics or NC transfection using Wound scratch assay; (E) cell invasion was determined using Transwell assay with Matrigel in hTERT-AM cells transfected with miR-546b mimic or miR-NC. Values presented are the mean ± standard (SD) from three independent experiments (*, P<0.05, **, P<0.01, ***, P<0.001). Scale bar: 100 μm.
in tumor initiation and progression through post-transcriptional regulation of gene expression (17). Previous studies have shown that miR-516b is expressed at low level in non-small cell lung cancer (NSCLC) (14), primary melanoma (15), and prolactinomas (16). However, little is known about the functional role and mechanistic action of miR-516b in AM. In the present study, we demonstrated that miR-516b was downregulated in AM tumor samples as compared to corresponding adjacent normal tissues. Also, we examined the effects of miR-516b on AM cells in vitro.

Our results indicated that overexpression of miR-516b significantly suppressed cell growth by inducing cell cycle arrest and apoptosis and inhibited cell migration and invasion capacity of AM cells, and restoration of MYCBP partially rescued the inhibitory effect of miR-516b on AM cells. Therefore, we opined that miR-516b plays a critical role in the suppression of proliferation and metastasis of AM cells by downregulating the expression of c-mycbp.

FIGURE 3 MYCBP is a direct target gene of miR-516b in ameloblastoma (AM). (A) Predicted sequence of miR-516b-binding site within the 3’UTR of MYCBP BY TargetScan; (B) luciferase assay was performed to confirm the direct regulation of miR-516b on the 3’UTR of MYCBP. ***, P<0.001 vs. NC; (C) analysis of c-mycbp protein levels in AM tissues by Western blotting, and protein quantification was analyzed according to the protein gray values; GAPDH was used as an internal control (*, P<0.05); (D) analysis of c-mycbp protein levels in hTERT-AM cells by Western blotting, and protein quantification was analyzed according to the protein gray values; GAPDH was used as an internal control (**, P<0.01). 3’UTR, 3’ untranslated region; wt, wild type; mut, mutant type.
Figure 4 Effect of MYCBP depletion on proliferation, cell cycle, apoptosis, migration, and invasion of ameloblastoma (AM) cells. (A) CCK-8 assay was used to determine cell proliferation in hTERT-AM cells after siMYCBP or scramble siRNA transfection; (B) representative cell cycle profile and quantification of hTERT-AM cells after siMYCBP or scramble siRNA transfection, detected using flow cytometry with PI staining; (C) apoptotic status after transfection, as detected by flow cytometry with Annexin-FITC/PI double staining; (D) images and quantification of the migration distances in hTERT-AM cells after siMYCBP or scramble siRNA transfection using Wound scratch assay; (E) cell invasion was determined using Transwell assay with Matrigel in hTERT-AM cells transfected with after siMYCBP or scramble siRNA transfection. Values presented are the mean ± standard (SD) from three independent experiments (*, P<0.05, ***, P<0.001). NC, cells transfected with scramble siRNA. Scale bar: 100 μm.
Figure 5 Effect of MYCBP overexpression on proliferation, cell cycle, apoptosis, migration, and invasion of v cells after transfection with miR-516b mimics. (A) representative cell cycle profile and quantification of hTERT-AM cells after pcDNA/MYCBP or pcDNA blank vector was transfected into miR-516b mimics cells, detected using flow cytometry with PI staining; (B) apoptotic status after transfection, as detected by flow cytometry with Annexin-FITC/PI double staining; (C) images and quantification of the migration distances in hTERT-AM cells after pcDNA/MYCBP or pcDNA blank vector was transfected into miR-516b mimic cells using Wound scratch assay; (D) cell invasion was determined using Transwell assay with Matrigel in hTERT-AM cells after pcDNA/MYCBP or pcDNA blank vector was transfected into miR-516b mimic cells. Values presented are the mean ± standard (SD) from three independent experiments (*, P<0.05, **, P<0.01, ***, P<0.001). NC, cells transfected with miR-NC or scramble siRNA; MYCBP, pcDNA/MYCBP. Scale bar: 100 μm.

MYCBP depletion. The findings confirmed the hypothesis of indirect regulation of c-myc activity by miR-516b through modulating the expression of its cofactor, MYCBP. RECK has been found to function as a tumor suppressor in many types of cancer (26-28). MMPs are extracellular matrix-degrading related enzymes that play a key role in tumor growth and invasion. MMP2 and MMP9 are the two important members of MMPs, which are inhibited by RECK at the post-transcriptional level (29,30). A recent study has showed that RECK is significantly downregulated in AM and its overexpression reduces invasive ability of AM cells by reducing the expression of MMP2 and MMP9 (31). In this study, it was observed that treatment with miR-516b mimic or siMYCBP could significantly inhibit c-myc
expression in order to decrease the expression of MMP2 and MMP9. It was observed that RECK, MMP2, and MMP9 function as downstream effector proteins of MYCBP, which are indirectly regulated by miR-516b.

**Conclusions**

Our results suggest that miR-516b function as a potential target for tumor suppression by exerting its inhibitory effect on proliferation, migration, and invasion of AM cells by indirectly regulating c-myc/RECK/MMPs pathway by inducing MYCBP expression. However, further studies are required to validate its role in tumor suppression in AM.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study and the samples used were approved by the ethics committee of Sun Yat-sen Memorial Hospital and written informed consent was obtained from all patients.

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