Tropical Journal of Pharmaceutical Research June 2021; 20 (6): 1113-1118
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v20i6.2

Original Research Article

MiR-497-5p promotes osteogenic/odontogenic differentiation of stem cells from the apical papilla by regulation of the TGF-β Smad pathway through Smurf2

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Sent for review: 18 March 2021 *Revised accepted: 29 May 2021*

Abstract

Purpose: *To study the influence of miR-497-5p on osteogenic/odontogenic differentiation (OOD) of SCAP, and the signal route involved.*
Methods: *Four groups were set up: miR-497-5p overexpression group (OEG), overexpression control OEC), miR-497-5p inhibition group, and inhibition control group. Alkaline phosphatase (ALP) activity was assayed, and calcified nodules measured. Protein expression levels of dentine salivary phosphoprotein (DSPP), collagen type I, ALP, osteoblast-related factors (Runx2, OSX and OPN) were also assayed. The mRNA expression levels of osteogenesis/dentin-related genes were determined.*
Results: *ALP activity was significantly higher in miR-497-5p overexpression cells than in control, but was reduced, relative to inhibition control group ($p < 0.05$). The miR-497-5p OEG had significantly more calcified nodules than OEC ($p < 0.05$). There were markedly up-regulated protein expressions in cells of miR-497-5p OEG than in OCG. Furthermore, protein expressions of Smad2, Smad3 and Smad4 in cells of miR-497-5p OEG were significantly up-regulated, relative to those in OEC, but wer lower in miR-497-5p inhibitory cells than in inhibitory cells.*
Conclusion: *MiR-497-5p enhances the OOD of SCAP via a mechanism involving TGF-β Smad pathway and Smurf2. Thus, Mir-497-5p may be used as a target for OOD-related drugs.*

Keywords: *MiR-497-5p, Smurf2 regulation, TGF-β Smad route, Stem cells, Apical papilla (SCAP), Osteogenic/ odontogenic differentiation*

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INTRODUCTION

Dental caries and dental trauma are common and frequently-occurring diseases in adolescents. If left untreated for long, these diseases may result in pulp necrosis or periapical lesions, blockage of the development of permanent teeth, and weakening of tooth resistance, which may ultimately affect the chewing capability and aesthetics of patients [1]. The SCAPs are cells present in the apical papilla of young permanent teeth. These cells have high proliferation and multi-directional differentiation capacity: they can differentiate into odontoblast-like cells and generate dentin, and they express osteogenesis and odontogenesis differentiation markers such as dentine sialo-phosphoprotein (DSPP) and alkaline phosphatase (ALP) [2].

MicroRNAs (miRNAs) are non-coding small RNAs involved in cell development, differentiation, migration and apoptosis [3]. Previous studies have shown that miRNAs are important in regulation of osteogenic differentiation of mesenchymal stem cells, most of which are regulated through signaling pathways [4]. Indeed, miR-497-5p is significantly increased in the differentiation of SCAP osteoblasts/odontoblasts [5]. In this study, the effect of miR-497-5p on OOD of SCAP, and its related mechanism were determined using transient transfection of SCAP with miR-497-5p.

EXPERIMENTAL

Equipment and reagents

The main reagents and equipment used in this study, and their sources (in parenthesis) were: α-MEN medium (Amyjet Scientific Ltd), fetal bovine serum (Wuhan Procell Life Technology Co, Ltd), Alizarin red (Shanghai Baoman Biotechnology Co. Ltd), anhydrous ethanol (Shanghai Zhenyu Biotechnology Co. Ltd), alkaline phosphatase staining kit (G-clone, Beijing Biotechnology Co. Ltd), and Lipofectamine 2000 (Shanghai Weijin Biotechnology Co. Ltd). The other reagents were MiR-497-5p or 10 μL miR-497-5p inhibition/ control, and fluorescent quantitative PCR detection kit (Biomics Biotechnology, Inc.); BCA protein concentration test kit (Beijing Kangjia Hongyuan Biotechnology Co. Ltd), anti-DSPP antibody (Shanghai Kemin Biotechnology Co. Ltd); anti-collagen Ⅰ antibody (Shanghai Yaji Biotechnology Co. Ltd), anti-human ALP and osteoblast-related factor (Runx2, OSX and OPN) antibodies (Amyjet Scientific Ltd.), and anti-human GAPDH antibody (Shanghai Huzhen Industrial Co. Ltd). The main instruments used were inverted fluorescence microscope, automatic cell counter, micropipettes and dry thermostat (Shanghai Fuze Trade Co. Ltd); ultra-low temperature refrigerator (Wuxi Lifes Biological Laboratory Equipment Co. Ltd); ultrasonic cell crusher (Shanghai Xinyu Biotechnology Co. Ltd), automatic chemiluminescence gel imager (Alit Life Sciences Co. Ltd), and SDS-PAGE electrophoresis and membrane transfer instrument (Shanghai Aiyan Biotechnology Co. Ltd).

Transient transfection of miR-497-5p

Cells from the apical papilla (SCAP) at logarithmic growth phase were maintained in a medium containing 10 % FBS without double antibody, and divided into miR-497-5p overexpression group, overexpression control group, miR-497-5p inhibition group, and inhibition control group. The experiment was carried out in a 6-well plate, with 3 repeated wells in each group. The cells were subjected to tryptic digestion and centrifuged at 1000 rpm for 5min. The supernatants were discarded, and the cells were resuspended at a density of 4×10^5 cells/mL in a medium containing 10 % FBS without double antibody, followed by incubation in a 5 % CO_2 incubator at 37 ^oC.

The transfection reagent Lipofectamine 2000 was diluted with Opti-MEM medium without double antibody and serum (250 μL Opti-MEM + 5 μL Lipofectamine 2000 per well), with thorough mixing, and stood at laboratory temperature for 5 min. The MiRNA was diluted with medium, and the dose per well was 250 μL Optimem + 5 μL miR-497-5p overexpression/control or 10 μL miR-497-5p inhibition/control, with thorough and gentle mixing, after which it was allowed to stand at room temperature for 20 min. Then, it was added to a 6-well plate so that the liquid evenly covered the bottom of the plate. Then, 4×10^5 cells were added to each well. The cells were uniformly distributed by shaking the 6-well plate, and cultured in 5 % CO_2 atmosphere for 6 h at 37 ^oC, after which the medium was replaced with complete medium.

Real-time fluorescence quantitative PCR

The original medium was discarded, and the cells were washed thrice with PBS buffer. Then, 1mL of RNAiso Plus was added to each well and evenly distributed on the cell surface. The cells were blown with a pipette and the lysates were transferred to 1.5-mL EP tubes to which 200 μL chloroform was added, shaken for 20 sec and allowed to stand for 5 min. Each mixture was clarified by centrifugation in the cold, and the supernatant taken up in a new EP tube was diluted with the same volume of isopropanol, followed by gentle shaking for thorough mixing, and keeping at room temperature for 10 min. The mixture was spun at 12000 rpm for 10 min at 4 ^oC, after which 1mL of 75 % ethanol was added to each EP tube, followed by centrifugation as before. The precipitate was solubilized in water free from RNase, and the Nano Drop1000 spectrophotometer was used to measure the concentration and purity of the RNA. The genomic DNA removal reaction and reverse transcription reaction were performed on ice, followed by real-time fluorescence quantitative PCR. After amplification, the fusion curve and amplification curve were used for quantitative analysis.

Assay of alkaline phosphatase (ALP) activity

After transfection, the culture medium was replaced with osteogenic induction solution after 24 h. Then, the cells were cultured for 7 days, and treated with PMSF-tainted RIPA lysis solution. The lysis was enhanced via ultrasound, and the lysate was centrifuged at 12000 rpm for 15 min at 4 °C. The protein concentration of the supernatant was determined colorimetrically, and the optical density was measured at 520 nm in a microplate reader. Each assay was done in triplicate, and the activity of ALP was calculated.

Alizarin red staining

After transfection, the culture medium was replaced with osteogenic induction solution after 24 h. Thereafter, the original culture medium was discarded, and the cells were PBS-rinsed, fixed with 4 % paraformaldehyde for 30 min, and rinsed thrice with PBS buffer, followed by addition of 1 mL alizarin red dye and incubation at 37 °C for 30 min. Then, the staining solution was removed, followed by cell rinsing thrice in PBS buffer. The results of staining were examined microscopically and pictured.

Western blot assay

After transfection, the culture medium was replaced with osteogenic induction solution after 24 h. Then, 200 µL of RIPA lysis buffer containing PMSF was used to lyse the cells in each well, on ice for 30 min, and the lysate was taken up in 1.5-mL EP tube. The lysis was enhanced with ultrasound, and the lysate was centrifuged at 12000 rpm for 15 min at 4 °C, followed by assay of lysate protein level with BCA method. Then, the proteins were resolved with SDS-PAGE and transferred to PVDF membrane which was subsequently incubated with 5 % fat-free milk solution to block non-specific blot binding. Membrane incubation overnight at 4 °C with appropriate 1° antibodies was followed by incubation with HRP-linked 2° antibody. Then, enhanced chemiluminescence and Grayscale analysis of the protein bands were carried out.

Statistics

All measured data are presented as mean ± SD, and were compared between groups using *t*-test. Counted data are expressed as percentages, and were comparted with χ^2 test. All statistical analyses were done with SPSS20.0 software package. Values of $p < 0.05$ indicated statistically significant differences.

RESULTS

ALP activity

Figure 1 shows that ALP activity was markedly higher in miR-497-5p OEG than in overexpression control (OEC), but was more markedly reduced in miR-497-5p inhibition group than in inhibition control ($p < 0.05$).

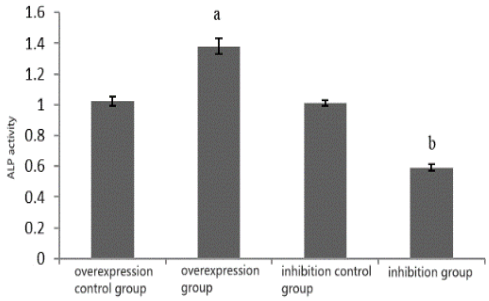

Figure 1: ALP activity in each group of cells. $^{a}P < 0.05$, vs overexpression control group; $^{b}p < 0.05$, vs inhibition control group

Calcified nodules

Figure 2 indicates that calcified nodules in the miR-497-5p were more numerous in overexpression cells than in OEC cells, but fewer in the inhibition group than in inhibition control cells ($p < 0.05$).

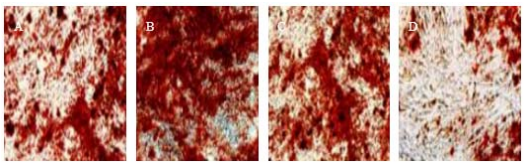

Figure 2: Calcified nodules in the cells

Osteogenic/odontogenic genes

The results of western blotting (Figure 3) showed markedly higher protein expressions of OOG genes in cells of miR-497-5p OEG than in cells of overexpression control, but they were markedly down-regulated in miR-497-5p inhibition cells than in cells of inhibition control ($p < 0.05$).

Protein levels of Smad

Protein expressions of Smad-2, Smad-3 and Smad-4 were markedly higher in cells of the miR-497-5p overexpression group than in OEC cells, but were markedly lower in cells of the miR-497-5p inhibition group than in cells of the inhibition control ($p < 0.05$; Figure 4).

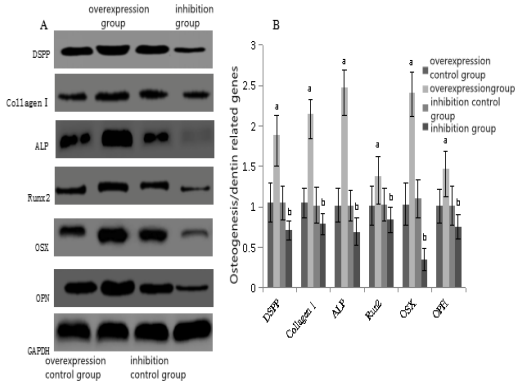

Figure 3: Osteogenic/odontogenic genes in the cells. (A) Expression levels of osteogenic/odontogenic proteins in each group of cells. (B) Comparison of osteogenic/odontogenic genes amongst the groups of cells. $^{a}P < 0.05$, vs overexpression control; $^{b}p < 0.05$, vs inhibition control

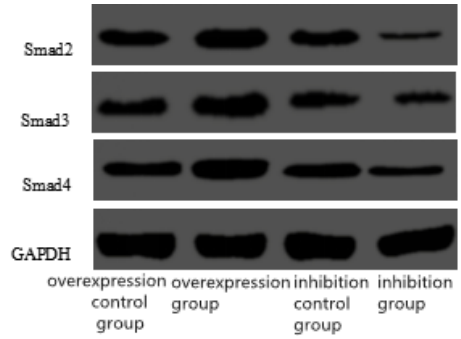

Figure 4: Smad protein expression level in the cells

Changes in osteogenic/odontogenic-related proteins after miR-497-5p suppression and interference with Smurf2

Inhibition of endogenous miR-497-5p and interference with Smurf2 in SCAP revealed that miR-497-5p facilitated OOD via regulation of TGF-β Smad signal route through Smurf2, thereby promoting OOD of SCAP. These results are shown in Figure 5.

DISCUSSION

Dental caries and dental trauma are common causes of stunted development of young permanent teeth in adolescents. Stem cells from the apical papilla (SCAP), as ideal seed cells for dental tissue engineering, have attracted a lot of attention from researchers in China and elsewhere, due to their good potential for proliferation and differentiation, and great application prospect in biological tissue engineering. They are also the focus of research on tooth regeneration [6].

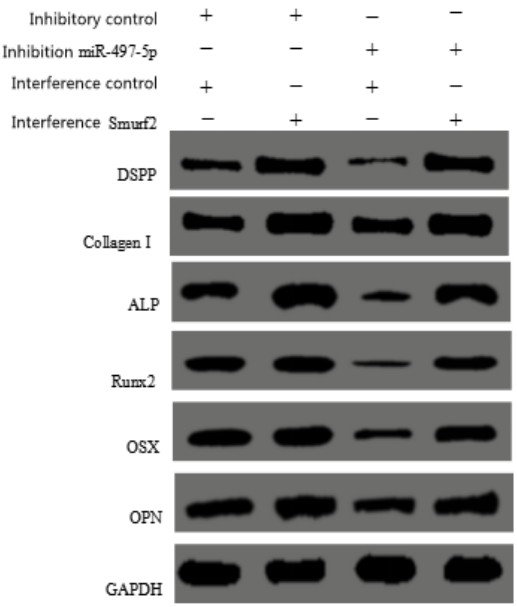

Figure 5: Verification of the expression of osteogenic/odontogenic-related proteins with Western blot

It is known that miRNAs are non-coding RNAs involved in the proliferation, differentiation and apoptosis of various cells due to their special hairpin structure [7]. Studies have shown that miRNAs activate and regulate potential pluripotency of human-derived mesenchymal stem cells, especially in the multidirectional differentiation ability of mesenchymal stem cells [8].

It has been reported that miR-199a-3p regulated adipogenic specialization of multipotent stem cells found in bone marrow via targeted regulation of KDM6A [9]. Moreover, a study showed that miR-143 inhibited osteogenic differentiation of dental pulp stem cells by regulating TNF-α [10]. Although MiR-497-5p is present in liver cancer, esophageal cancer, breast cancer and bladder cancer, the role of SCAP is not yet fully understood. Runt-2 is a multifunctional transcription factor/protein that plays an important role in differentiation and bone formation of osteoblasts [11], while DSPP is an odontogenic non-collagen protein expressed by odontoblast cells, and is a relatively recognized tooth-specific protein [12].

Collagen I is an important part of the extracellular matrix of dentin, and changes in its expression reflect cell differentiation and maturity state of tissue cells. Alkaline phosphatase (ALP), an enzyme protein secreted by osteoblasts during early proliferation, promotes the

mineralization of osteoblasts, and it is one of the specific markers of differentiation of osteoblasts. One of the most abundant non-collagenous bone proteins is OCN specifically expressed in the late stage of osteoblast differentiation. It regulates the deposition and metastasis of bone minerals, and promotes the differentiation and maturation of osteoblasts and the formation of osteocytes. It is known that OPN is a non-collagenous bone matrix glycoprotein expressed by osteocytes, osteoblasts, osteoclasts and chondrocytes, and it is important in the mineralization of bone matrix.

The present study showed that ALP activity, calcified nodules, and OOD gene and protein levels in cells of the miR-497-5p overexpression group were markedly higher than those in cells of the overexpression control group, but they were lower in cells of the miR-497-5p inhibition group than in inhibition control cells. These findings indicate that miR-497-5p significantly improved OOD of SCAP, but the mechanism remains unclear. These findings are similar to the results reported in a previous study [13].

A Smad-specific E3 ubiquitin protein ligase 2, Smurf2 is an important component of the ubiquitin-protease degradation system, and it is involved in the regulation of signaling factor transduction of TGF-β family. Previous studies have confirmed that TGF-β Smad pathway is involved in a series of biological processes such as cell growth, differentiation and apoptosis [14].

The TGF-β signal transduction in cells is mainly transmitted by Smad family of proteins which are important in the transmission of TGF-β signal from cell membrane to cytoplasm. In particular, Smurf2 interacts with Smurf3 and Smurf7, and it also regulates signal transduction by combining with Smurf4 in an indirect way. Therefore, Smurf2 performs a key function in degradation of TGF-β Smad pathway through the ubiquitin-proteasome pathway [15-17].

In the present study, the protein expressions of Smad2, Smad3 and Smad4 in cells of the miR-497-5p overexpression cells were markedly up-regulated, relative to the overexpression control, but they were markedly lower in cells of the miR-497-5p inhibition group than in cells of the inhibition control. Thus, miR-497-5p effectively regulated the TGF-β Smad pathway. By analyzing the osteogenic/odontogenic related proteins after suppression of endogenous miR-497-5p and interference with Smurf2, it was revealed that miR-497-5p promoted OOD by regulating TGF-β Smad route through Smurf2, thereby enhancing the OOD of SCAP.

CONCLUSION

The findings of this study indicate that MiR-497-5p promotes OOD of SCAP through the regulation of TGF-β Smad route and Smurf2. Thus, Mir-497-5p may serve as a target for OOD-related drugs.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jie Chen designed the study, supervised the data collection, and analyzed the data. Limiao Zhang interpreted the data and prepared the manuscript for publication. Chenyan Liu and Yuelei Liang supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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