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Original Article

Downregulation of microRNA-216 in nucleus pulposus cells contributes to vertebrae disruption via targeting RANK in lumbar disc herniation

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Abstract: Lumbar disc herniation (LDH) is a common cause of lumbosacral radiculopathy, and vertebral body or endplate destruction is often seen in LDH patients in clinic. High osteoclastogenesis activity occurs in the progression of LDH but the mechanisms are poorly understood. MicroRNAs (miRNAs) have become increasingly responsible for important gene regulatory functions in various contexts. However, the function of miRNA on osteoclast differentiation in LDH is still unknown. In the current paper, we found that expression of miRNA-216 (miR-216) was significantly decreased in nucleus pulposus cells of LDH patients compared with health donors. Coincidentally, miR-216 seed sequences were complementary to a sequence conserved in the 3'-untranslated region (3'UTR) of receptor activator of nuclear factor kappa-B (RANK) mRNA. We induced osteoclast (OC) cells from monocytes in vitro and found miR-216 expression decreased gradually during OC differentiation. Western blot analysis showed that miR-216 remarkably suppressed the RANK protein level, and the post-transcriptional repression of RANK by miR-216 was further confirmed by the luciferase reporter assay. As a result, RANKL-induced osteoclast differentiation was significantly attenuated when synthesized miR-216 was presented, and the RANKL-RNAK-NF-κb pathway was greatly inhibited. Therefore, these results showed that the downregulation of miR-216 in nucleus pulposus cells might promote osteoclastogenesis activity by increasing the expression of RANK, which results in vertebral bone destruction.

Keywords: Lumbar disc herniation, osteoblasts differentiation, miR-216, RANK

Introduction

Lumbar disc herniation (LDH), which is a common disease in elderly patients, is defined as the narrowing of the spinal canal with cord or nerve root impingement causing the occurrence of radiculopathy or pseudoclaudication [1]. During the LDH progression, bone remodeling is a physiological process of repeated activation-resorption-formation cycles that involve activation of osteoclasts to resorb bone and osteoblast-mediated new bone formation [1]. Osteoclasts, generated by differentiation of monocyte/macrophage hematopoietic lineages, are bone-specific multinucleated cells and excrete lytic enzymes to degrade bone matrix. The target to treat bone-lytic damage in LDH by regulating osteoclast differentiation may be a potential strategy [2, 3].

Osteoclast formation is stimulated by receptor activation of nuclear factor κB ligand (RANKL), which is present as a membrane-bound protein on the surface of osteoblasts [4]. The effects of RANKL are influenced by RANK, a specific receptor; genes are engaged in osteoclast differentiation and activation and either up- or down-regulated [5]. NF-κb was the downstream signaling pathway for RANKL-RANK, and RANKL-RANK-NF-κb interactions were essential for osteoclast formation [6]. Whereas, how this signaling pathway was regulated in the process of osteoclast formation in LDH is largely unknown.

MicroRNAs (miRNAs) which are a class of about 22 nucleotide non-coding RNA molecules that negatively regulate gene expression at the post-transcriptional levels. MiRNAs are one of the largest classes of gene-regulatory molecules

which could affect cell proliferation, differentiation, cell death, and most other biological processes [7]; miRNAs not only regulate normal physiological processes, but also function in the development of most disorders, including LDH. There were few researches focused on the function of miR-216 in the biological process. MiR-216 was firstly detected in 2007, and the papers were mainly about the expression change of miR-216 in pancreatic cancer patients [8-10]. The expression level change and function of miR-216 in osteoclasts differentiation of LDH is incompletely understood.

In this study, we aimed to explore the potential role of miR-216 in the progress of osteoclasts differentiation in LDH. We compared the expression of miR-216 and other osteoclasts marker genes in tissues from LDH patients, such as CALCR, CTSK and TRAP. Next, we investigated the correlation between miR-216 and other marker genes respectively. Furthermore, the in vitro cell model was constructed and the expression of miR-216 and other genes were also verified. Finally, we examined the function of miR-216 on cell apoptosis and detected the expression of osteoclasts marker genes in vitro.

Materials and methods

Patients samples

LDH specimens from 125 patients who had received surgical therapy between October, 2012 and June 2014 were obtained through primary lumbar discectomy for radiculalgia from the Department of Orthopedics and Spine in the Tengzhou People's Central Hospital. (LDH group, 56 men and 69 women with mean age 52.20±9.57 years); 27 traumatism donors (Control group, 14 men and 13 women with mean age 29.56±6.72 years) was used as the normal control. Diagnosis criteria were as previously reported [11]: briefly, available magnetic resonance imaging (MRI) and computed tomography (CT) scan demonstrating LDH corresponding to the neurological level and side suggested at the clinical presentation. The study was in accord with the ethical standards established by the institution or in accord with the Helsinki Declaration. The study protocol was approved by the Ethics Committee of Medical School of Shandong University (Jinan, Shandong, China).

Osteoclast differentiation and TRAP staining

Protocols have been described in a previous study [11]. Briefly, Peripheral blood mononuclear cells (PBMCs) were pated in 24-well plates and allowed to adhere for two hours in RPMI-1640, then the adherent monocytes cells were cultured in α-MEM (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Hyclone, CA, USA) and 25 ng/mL human M-CSF (R&D Systems, NY, USA). After twelve days of culture, TRAP was stained using a leukocyte acid phosphatase kit (Sigma-Aldrich, CA, USA) following the manufacturer's instructions. Briefly, differentiated osteoclast cells were fixed with citrate/acetone solution and stained with acetate/naphthol/tartrate solution at 37°C for one hour, and the nuclei were stained with hematoxylin at room temperature for 5 minutes. Differentiated cells with characteristics of TRAP staining positive, three or more nuclei and a cell body larger than 100 mm were considered to be osteoclasts.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The analysis of mRNA expression was performed using a Bio-Rad iCycler (Bio-Rad Laboratories, CA, USA), and each mRNA expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ribosomal RNA expression for each sample. Briefly, total RNA 1 μg was used in the reversal transcription (RT) reaction with 0.5 μg of oligodT primer, 10 mmol/L of each of the 4 dNTP, 25 mmol/L of $MgCl_2$, 10 U of RNA inhibitor and 50 U of superscript II reverse transcriptase (all purchased from Invitrogen, CA, USA) according to the manufacturer's instruction. The final solution was used directly for PCR amplification. Each cDNA reaction was diluted, along with a calibrator sample containing the transcript of interest and run in 25 μL of Bio-Rad SYBR Green (Bio-Rad Laboratories, CA, USA) reactions using 10 pmol of primers. Reactions were processed using one initial denaturation cycle (5 min at 94°C), then 30 cycles of denaturation (30 s at 95°C), annealing (45 s at variable for gene) and amplication (1 min at 72°C) followed by melt curve determination consisting of one denaturation cycle (1 min at 95°C), annealing (one cycle for 1 min at 55°C) and then 80 cycles (5 s each at 55°C-95°C). Human gene-specific oligonucleotide sequences were as follows: *CALCR,*

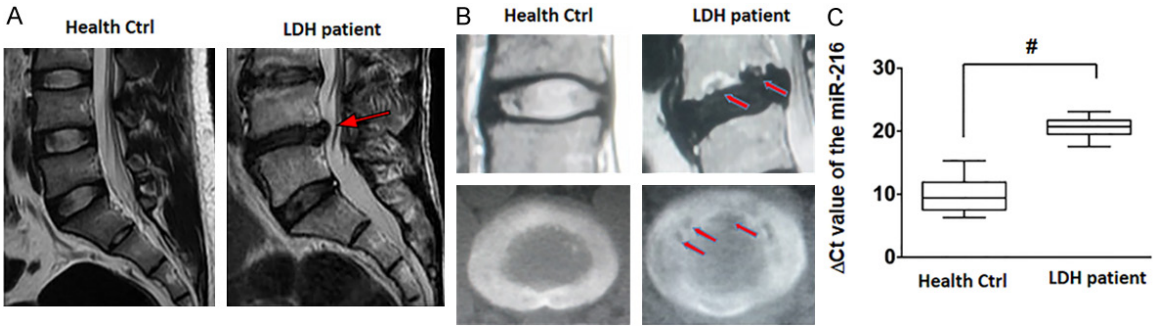

Figure 1. MiR-216 level in nucleus pulposus cells from LDH patients. A. Representative images of MRI T2 in healthy control and LDH patients showing the herniated lumber disc (red arrow). B. Representative images of CT scan in healthy control and LDH patients showing the destructed vertebra body (red arrows) in the LDH patients. C. Real time PCR showing the miR-216 levels in the healthy subjects group and LDH patients with vertebra bone destruction. ΔCt value represents the Ct^{LDH}-Ct^{Ctrl}, and a higher ΔCt value comes with a lower expression level. #, P<0.001.

TTACCCGCATACCAAGGAGAA, TGGGCAGAACTGATAGGACAATA; *CTSK*, GCAGAAGAACCGGGGTATTGA, GAAGGAGGTCAGGCTTGCAT; *TRAP*, GACTGTGCAGATCCTGGGTG, GGTCAGAGAATACGTCCTCAAAG; *GAPDH*, ACAACTTTGGTATCGTGGAAGG, GCCATCACGCCACAGTTTC.

Western blot

Cultured cells were lysed in 1× lysis buffer (Cell Signal Technology, CA, USA) containing protease inhibitors (1× PMSF and 1× cocktail). Equivalent amounts of protein were separated by electrophoreses and transferred onto a polyvinylidene fluoride (PVDF) Membrane (Millipore, MA, USA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline and then incubated with CALCR, CTSK, TRAP, NF-κB or phosphor-NF-κB monoclonal antibodies (Santa Cruz Biotech, CA, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotech, CA, USA). GAPDH (Santa Cruz Biotech, CA, USA) was used as a loading control.

Luciferase report assay

1013 bp of the RANK mRNA 3'UTR fragment containing miR-216 binding sites was cloned into the pMIR-REPORT-luciferase plasmid (Promega, Madison, WI, USA) by PCR with the primers containing SpeI and HindIII restriction enzymes sites (pMIR-RANK-3'UTR wt): F: 5'-GACTAGTAAATGGGCTTTTCAGGAAGTG-3'; R: 5'-CAAGCTTGTCCATCTTTTCCAGAGAGACCT-3'. Mutation of the miR-216 sites at the wild type pMIR-RANK-3'UTR were performed by TransGen (Beijing TransGen Biotech, Beijing, China). For luciferase reporter assays, HeLa cells were seeded in 48-well plates. MiR-216 mimics and sequence scrambled RNA controls were co-transfected with luciferase reporter vectors by using lipofectamine 2000 (Invitrogen, CA USA). Two days later, cells were harvested and assayed with the Dual-Luciferase Assay (Promega, Madison, WI USA). Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC).

Statistical analysis

All statistical analyses of data from both groups were conducted by the SPSS system (SPSS Inc., Version 18.0. and Chicago, USA). Mann-Whitney U-test was used to assess the difference in miR-216 concentration between Con group and LDH group. All data are expressed as mean ± standard error of the mean. The difference between groups was considered significant when P<0.05.

Result

MiR-216 expression was decreased in patients samples

Diagnosis of LDH patients and inspection of vertebra body disruption were made according to clinical appearance and confirmed by magnetic resonance imaging (MRI) examination and computed tomography (CT) scan as previously described [11]. As shown in the **Figure 1**, the representative images of patients with lumbar disc herniation demonstrated a mass in the

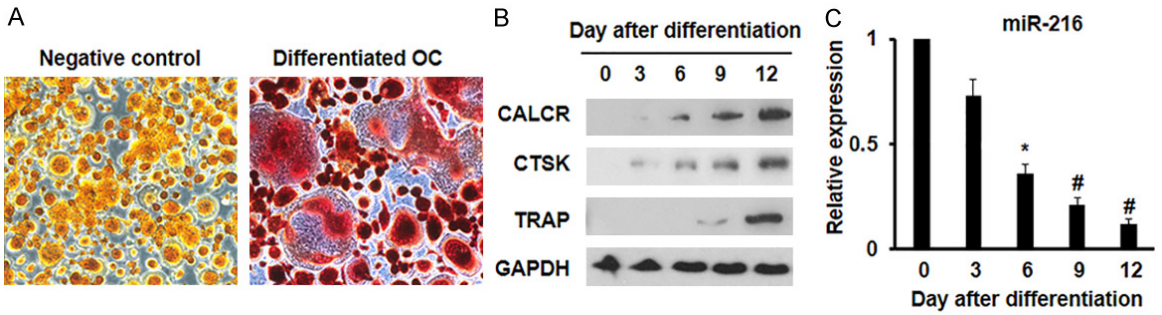

Figure 2. MiR-216 expression decreases in osteoclast differentiation. A. TRAP staining showing the morphology of mature osteoclast cells in a monocytes induction model using RANKL (50 ng/ml) and M-CSF (25 ng/ml) for 12 days. Media was changed every 3 days. Mature OC cells were defined as multinuclear (>3), plasma TRAP+, and body size >100 μm cells. B. The expression of CALCR, CTSK and TRAP were determined via western blot during osteoclast differentiation, the expression level of these protein in day 0 were used as the control respectively. C. The expression of miR-216 was determined via qRT-PCR during osteoclast differentiation, the expression level of miR-216 in day 0 was used as the control. *, P<0.05 compared with control; #, P<0.001 compared with control.

posterior epidural space at the L3-L4 level (red arrow), but there was no dissection textural anomaly observed in the healthy control subjects (**Figure 1A**). Bone lesion on vertebra body exhibited low intensity on T1-weighted MRI (red arrows) (**Figure 1B**).

To explore the detailed molecular mechanism in the progress of vertebral bone destruction in LDH patients, nucleus pulposus samples from the patients were procured for RNA extraction, and the miR-216 expression level was detected via qRT-PCR assay. The results showed that miR-216 was significantly downregulated in LDH patients samples compared with control group (**Figure 1C**). Therefore the correlation between miR-216 and LDH was validated.

MiR-216 expression was decreased during osteoclast differentiation

In order to verify whether miR-216 plays any role in osteoclast differentiation, osteoclast (OC) differentiation was induced from CD14+ monocytes from the peripheral blood mononuclear cells (PBMC), and the expression of miR-216 was examined during the differentiation. In the presence of 25 ng/ml of RANKL and 50 ng/ml of M-CSF, CD14+ monocytes first developed into preOCs in a 6-day culture, and then developed into mature OCs with RANKL after an additional 6 days of culturing [12]. Differentiated OCs were also determined by TRAP staining showing multinuclear (>3 nucleus per cell) and TRAP+ in the cell cytoplasm (**Figure 2A**). It was reported that during osteoclast differentiation, associated genes such as those for, calcitonin-related polypeptide alpha (CALCA), cathepsin K (CTSK) and tartrate-resistant acid phosphatase (TRAP) are encoded and expressed, which could be used as the marker for osteoclast differentiation [12]. We also detected at time point day 0, 3, 6, 9 and 12 in our experiments. Expressions of all these markers were observed from day 6, and increased gradually till the last day of induction (**Figure 2B**). On the other hand, the miR-216 expression was detected through qRT-PCR assay, the result showed that miR-216 was significantly downregulated following with the day of differentiation (**Figure 2C**). Thus, a negative correlation between miR-216 and osteoclastogenesis was established in our study.

RANK was the target gene of miR-216

As we all know, miRNAs usually exert their function via binding to the mRNA 3'UTR of the target genes. Through bioinformation prediction, we found that there were conserved binding sites on the mRNA of RANK for miR-216, which suggested that RANK might be the target gene of miR-216 (**Figure 3A** upper panel). In order to determine whether miR-216 direct targets RANK post-transcriptionally, we transfected miR-216 mimics in the differentiated OC cells, and western blot were employed to detect the RANK expression level. Our results showed that RANK protein level was remarkably decreased in cells transfected with miR-216 mimics compared with cells transfected with Scramble (**Figure 3B**). In addition, to investigate the direct

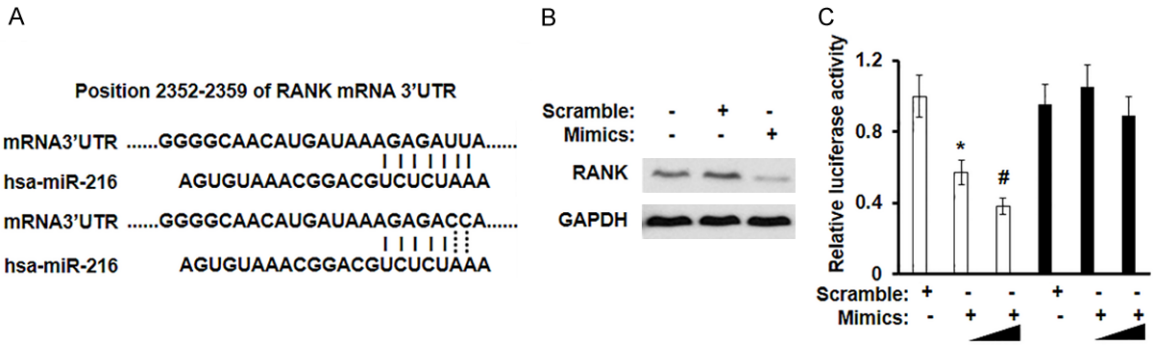

Figure 3. MiR-216 directly targets RANK mRNA 3'UTR. A. The information prediction of miR-216 binding sites on the 3'UTR of RANK mRNA and the mutation of 2 of these binding sites were shown. B. The expression of RANK protein was determined via western blot in cells transfected with Scramble or miR-216 Mimics in pre-OC cells (6 days after induction). The cells without transfection were used as the control. C. The luciferase reporter assay was employed to detect the luciferase activity in cells transfected with wild type RANK 3'UTR reporter plasmid or the mutated plasmid, with co-transfection with the Scramble and miR-216 Mimics (0, 50, 100 nM respectively). *, P<0.05 compared with control; #, P<0.001 compared with control.

binding of miR-216 on RANK mRNA 3'UTR, we cloned 1013 bp of the 3'UTR fragment of RANK mRNA containing miR-216 binding sites into the pMIR-REPORT-luciferase plasmid, as well as the mutant construct in which two of the miR-216 binding sites were changed (**Figure 3A** lower panel), and employed the luciferase reporter assay to detect the RANK mRNA transcriptional activity. The luciferase results showed that miR-216 could inhibit the luciferase activity in cells transfected with wild type RANK 3'UTR reporter plasmid in a dose dependent manner, whereas the phenomenon disappeared with mutate RANK 3'UTR reporter plasmid (**Figure 3C**), which indicated that RANK was the direct target of miR-216.

MiR-216 suppressed osteoclast differentiation via inhibition of NF-κB signaling

Given the results that miR-216 could direct target the mRNA of RANK, the function of miR-216 in the osteoclast differentiation was verified further. When miR-216 mimics or scramble were transfected into the monocytes then induced them to mature osteoclast cells using M-CSF and RANKL, the inhibition of miR216 on OC differentiation would be obviously seen in morphology (**Figure 4A**). As shown in the **Figure 4A**, TRAP+ multinuclear mature OC cells were successfully induced in the control group (OC Con), and there was no significant difference seen in the scramble control group (OC+Scr) compared with the OC control; however, there were obviously less mature OC cells found in the miR-216 mimic group (OC+Mimic). When quantified the OC cell numbers, we found generally about 120 mature OC cells seen per well of a 24-well plate, but only less than 70 mature OC cells seen in the miR-216 mimic group (P<0.01) (**Figure 4B**). Furthermore, the expression level of osteoclast differentiation markers were detected via qRT-PCR and western blot, the result showed that the overexpressed miR-216 significantly increased the mRNA and protein level of CALCR, CTSK and TRAP (**Figure 4C** and **4D** left panel). In addition, with the suppression of mi-216 on RANK expression, the NF-κB signaling pathway was significantly inhibited (**Figure 4D** right panel), which sequentially obstructed the osteoclast differentiation. Since miR-216 could inhibit osteoclast differentiation via blocking RANKL-RNAK-NF-κb pathway, decreasing expression level of miR-216 in LDH patients may contribute to the high osteoclast activity.

Discussion

Lumbosacral radiculopathy, a relatively common condition, is caused by mechanical deformation and biochemical irritation duo to intervertebral disc herniation [13]. LDH is the most common cause of sciatica, a condition that limits the daily activities severely [14]. Although the incidence of LDH is high in society, controversy over the pathogenesis and treatment of the disease still exists. Previous studies have detected the relation between osteoclast formation and LDH. According to the findings of

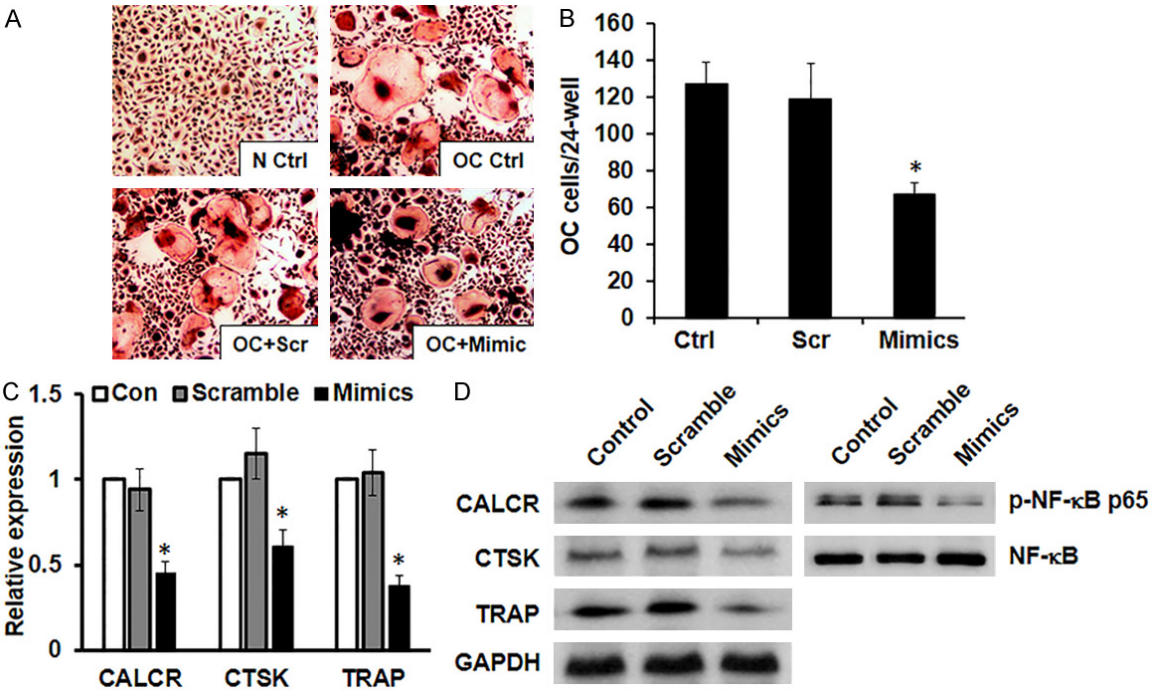

Figure 4. MiR-216 inhibits osteoclast differentiation via RANK-NF-κB signaling. TRAP staining showing the (A) morphology and (B) number of mature osteoclast cells in the normal osteoclast group, the miR scramble or miR-216 treated OC cells. (C) The expression of CALCR, CTSK and TRAP were determined via qRT-PCR in cells transfected with Scramble or miR-216 Mimics. The cells without transfection were used as the control. (D) The expression of CALCR, CTSK, TRAP and NF-κB protein were determined via western blot in cells transfected with Scramble or miR-216 Mimics. The cells transfected with Scramble was used as the control. *, P<0.05 compared with control.

those studies, the present study investigated the detailed molecular regulation of miRNAs in the osteoclast formation progress.

Perforation of bone tissue and intervertebral disc initiates bone repair and bone remodeling processes. In the early stages of healing in our experimental lesion activation of RANKL/RANK system and osteoclastogenesis is often observed. RANKL is a member of the tumor necrosis factor family, produced by osteoblasts and bone marrow stromal cells [15], and functions in osteoclast differentiation and activation [5]. RANK, as a receptor for RANKL, is expressed on the surfaces of pre-osteoclasts and plays an important role in osteoclastogenesis [16]. RANKL binds to RANK on osteoclast precursors and induces osteoclast differentiation in the presence of M-CSF [17]. Interestingly, Pettit *et al.* found that RANKL-knockout mice were protected from bone erosion in a serum transfer model of arthritis [18]. Multiple transcription factors including NF-κB, as the downstream of RANKL/RANK system, have been reported to determine osteoclast fate [19]. Therefore, it is plausible that control of the RANKL/RANK signaling pathway provides a way of suppressing osteoclast differentiation/activation. Nevertheless, how we fine-tune the RANKL/RANK signaling pathway is largely unknown.

miRNAs are important in many pivotal events regulating cell proliferation, differentiation, metabolism and apoptosis, and also involved in the pathogenesis of many disease [20]. So far, some relevant miRNAs have been found dysregulated in osteoclast differentiation. Pitari *et al.* showed that overexpression of miR-21 within multiple myeloma (MM) microenvironment plays a crucial role in bone resorption/apposition balance, supporting the establishment of miR-21 inhibition-based strategies for MM-related bone disease [21]. The study of Sun *et al.* revealed that miR-20a could regulate autophagy related protein-ATG16L1 by binding to its 3'UTR end in hypoxia-induced osteoclast differentiation [22]. M Baya-Moutoula *et al.* demonstrated that miR-223 affected not only the expression of its target genes NFIA and

RhoB, but also osteoclast marker genes and the Akt signaling pathway, which resulted in osteoclastogenesis [23]. Qu *et al.* revealed that miR-218 was acting as a negative regulator for osteoclastogenesis and bone resorption by suppressing the p38MAPK-c-Fos-NFATc1 pathway [24]. All together, these researches supplied promising therapeutic means against osteopenic diseases.

With respect to the functions of miR-216, there was nearly no research. Only Zhao *et al.* predicted many microRNA binding sites on the 3'-UTR of GABAA receptors, but not on GABAC receptors through computational approaches, and 3'-UTR reporter assays verified miR-216 as the microRNAs that target GABA receptor α1-subunit, which provides a basis for further studies of post-transcriptional regulation of GABA receptors [25]. In our study, we firstly identified RANK as a direct target of miR-216 through bioinformation prediction and solid molecular biology experiments. MiR-216 suppresses osteoclast differentiation and activity, therefore when in LDH patients the miR-216 level downregualted, suppression on OC activities is released increases leading to high OC activity. This hypothesis may explain why bone destruction is often seen in severe LDH patients. So, searching and confirming the target genes of miR-216 as well as the mechanism of its biological function in LDH patients has clinical merits.

Taken together, our results presents miR-216 is down-regulated in LDH patients compared with health donors, and also suppressed during the osteoclast differentiation, which indicates that miR-216 has a negative correlation with bone destruction of vertebrate body of LDH patients and plays a critical role in osteoclast differentiation. We further find that miR-216 functions through RANKL-RNAK-NF-κb pathway by targeting RANK. These results may provide a potential therapeutic target for osteoporosis.

Disclosure of conflict of interest

None.

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