DIFFERENTIAL EXPRESSION PROFILE OF MICRORNA IN BONE MARROW MESENCHYMAL STEM CELLS OF RATS WITH DIABETIC OSTEOPOROSIS

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Introduction

Diabetic Osteoporosis (DOP), due to the function impairment of bone marrow mesenchymal stem cells (BMMSCs), is commonly seen. The purpose of this study was to identify the different expressed microRNA (miRNA) in DOP BMMSCs.

Materials and methods: In our study, we used diabetic rats as models to explore the role of miRNA in DOP BMMSCs. First, the osteoporosis conditions of diabetic rats were assessed, then their BMMSCs were cultured to make the experimental group. At the same time, BMMSCs taken from normal rats were cultured as the control group. In the final stage, differently expressed miRNA were singled out using deep sequencing technology.

Results: The result showed that 8 weeks after the rats were induced to diabetes by streptozotocin (STZ) intraperitoneal injection, the density of their humerus was lower, their BV/TV (bone volume relative to tissue volume) was smaller and the trabecular spacing was greater than the wistar group. In addition, 10 pieces of miRNA were selected for their striking differences.

Conclusion: By the significant enrichment information in pathway, the Notch signal pathway could regulate the diabetic osteoporosis BMMSCs. Our study has provided new ideas in terms of a further discussion of how miRNA works in DOP.

Key words: diabetic osteoporosis (DOP), bone marrow mesenchymal stem cells (BMMSCs), microRNA.

DOI: 10.19193/0393-6384_2016_2_63

Received May 30, 2015; Accepted January 02, 2016

Introduction

Diabetic Osteoporosis (DOP) is a severe chronic complication of diabetes. With the aging of population as well as the increased incidence of obesity, the cases of DOP occur more often year by year. Epidemiological survey shows that among type I diabetes patients, the incidence of osteopenia and osteoporosis is 48-71%[1] while it is 20-60% among type II diabetes patients[2]. Under normal circumstances, the processes of osteogenesis and bone absorption are alternately happening within the body in a complex but orderly way, which makes the bone in the state of dynamic rebuilding. While in DOP patients, the balance between the two processes are broke, due to the function impairment of bone marrow mesenchymal stem cells (BMMSCs). Studies have found that when glucose concentration increases, the BMMSCs will show signs of early senescence, causing significant apoptosis and less proliferation[3], the activity of alkaline phosphatase (ALP) and the number of calcium nodules are all decreased significantly[4]. They believe that osteogenic potential of BMMSCs are decreased in high glucose condition.

Studies have found that some changes of microRNA (miRNA) expression profile will lead to distinct results of stem cells function. Li H has found in-vitro study that miR-2861 implemented a high expression particularly in bone tissues and it promoted osteogenesis differentiation by repressing histone deacetylase 5(HDAC5) expression[5].
Eskildsen et al. have discovered that miR-138 has the effect of inhibiting osteoblast formation in human BMMSCs. A recent study has found that by regulating SATB2 and Runx2, miR-205 influences the BMMSCs’ differentiation into osteoblasts. So far, as to DOP, the mechanism of how miRNA acts on stem cells to form osteoblasts is unknown, the regulating methods by target gene is also unclear. Therefore, we set up the DOP rat model to isolate and culture BMMSCs, use miRNA deep sequencing to seek for differently expressed miRNA, and then, based on biological information, make analysis and prediction of how miRNA regulates gene. By doing these experiments, we may discover the possible roles of miRNA in the onset of DOP.

Materials and methods

Experimental animals and modeling
The Animal Research Committee of Chinese PLA General Hospital, Beijing, China, approved all procedures. In the experiment, thirty-six 6-week-old male wistar rats (150±20g) of clean grade were randomly divided into 3 groups: the control group (n=12), the healthy group (wistar group, n=12) and the diabetic model group (diabetes group, n=12). All rats were fed adaptively for one week. Then control group was sacrificed. Diabetes group stopped feeding about 12 hours, and then was given intraperitoneal injection of streptozotocin (STZ, sigma, USA) at the level of 60mg/kg. In 72 hours, we took samples of the venous blood from their tails and measured the amount of glucose. If the random blood glucose was over 16.7mmol/L, the sampled rat was considered as a successful model; otherwise, the rats would be excluded from the experiment. The remained rats were routinely fed for 8 weeks and had their blood glucose measured each week.

MicroCT scanning of bone tissue density
The following samples were collected and preserved in 10% neutral formalin solution: humerus of the rats at the time before modeling (control group) and 8 weeks after modeling (wistar group, diabetes group). The samples were respectively scanned on the stage of Inveon MM CT (computed tomography) (SIEMENS, USA) (80KV, 500μA) at a resolution of 10.44 μm to get serial flat μCT images. The resolution of the images was 21 μm. After scanning, the following areas were selected as ROI (region of interests) to do 3-dimensional reconstruct: cancellous bone from 1 mm below the growth plate of proximal humerus to one mm distal humerus. Inveon Research Workplace (SIEMENS, USA) was employed to do morphometric analysis of bone tissues. The parameters included BMD (bone mineral density, mg/cm²), BV/TV (bone volume relative to tissue volume, %), BSA/BV (bone surface area relative to bone volume, 1/mm) and Tb.Sp (trabecula spacing, mm).

Cell culture
After the rats were executed by bloodletting at aorta abdominalis, the femur and tibia were harvested, and two ends of each bone were cut off to expose the marrow cavity. The marrow channels were flushed repeatedly by 5ml complete medium of wistar BMMSCs(Cyagen Biosciences, USA). Cells were collected and seeded onto a 25 cm² culture flask at a density of 5 × 10⁵/mL, and incubated in 5% CO₂ at 37 °C. The medium was changed every 3 days. The third generation was used to do the next experiment.

HiSeq Used to Test Sequence and Analyze Data
All RNA was extracted from 3rd logarithmic phased wistar-BMMSCs and diabetes-BMMSCs cells sample. The 49nt sequence tags from HiSeq sequencing went through the data cleaning analysis to get credible clean tags. Then the length distribution of the clean tags and common and specific sequences between samples had been summarized. Then the standard analysis annotated the clean tags into different categories and take those that cannot be annotated to any category to predict the novel miRNA and seed edit of potential known miRNA. After getting miRNA result, target prediction, differential expression, cluster analysis, GO enrichment and KEGG pathway for target genes were analyzed.

Statistical analysis
T-test and single factor analysis was used to compare two groups with Software SPSS 20.0 (SPSS Chicago, IL, USA). Data were expressed as mean±SD, p values of <0.05 were considered significant.

Results
Histomorphometric analysis using MicroCT
The microCT scan images of proximal humerus
three dimensional reconstruction results (Figure 1) demonstrated that rats’ proximal humerus structure of control group (7 weeks old) and wistar group (15 weeks old) were completed, while the rats’ proximal humerus of diabetes group were slim, discontinue, the amount was obviously reduced, and the cancellous bone in the humerus vanished.

Bone histomorphometric analysis showed that, compared with control group, the BMD of humerus in wistar group was significantly increased \((P<0.05)\), the BMD of humerus in diabetes group was significantly reduced \((P<0.05)\). Compared with wistar group, the humerus in diabetes group showed a significant reduction in BMD and BV/TV (bone volume relative to tissue volume), and a significant increase in Tb.Sp (trabecula spacing) \((M, N, O, P)\). \(^*\) means \(P<0.05\) VS wistar; \(^#\) means \(P<0.05\) VS control.

**HiSeq deep Sequencing and Data Analyzing**

Known miRNA expression between sample wistar and sample diabetes were compared by Expdiff to diagnose whether there have significant differences between two samples, and log2-ratioScatter plot (Figure 2) was used to compare the differences between miRNA expression quantity expressed by two samples. According to sort log2-ratio, ten differentially expressed miRNAs were filtrated (Table 1), of which 4 miRNA \(\text{rno-miR-146a-5p} \), \(\text{rno-miR-182} \), \(\text{rno-miR-672-5p} \), \(\text{rno-miR-99a-5p} \) were up-regulated from diabetes sample and 6 miRNA \(\text{rno-miR-10a-5p} \), \(\text{rno-miR-199a-5p} \), \(\text{rno-miR-340-5p} \), \(\text{rno-miR-298-5p} \), \(\text{rno-miR-351-5p} \), \(\text{rno-miR-106b-5p} \) were down-regulated.

Mainly biochemical metabolism and signal transduction pathways involved differentially expressed gene were confirmed by pathway significantly enrichment. In which Notch signal pathway had the most significant association with osteogenesis. According to the prediction of target gene, \(\text{rno-miR-10a-5p} \), \(\text{rno-miR-298-5p} \), \(\text{rno-miR-672-5p} \), \(\text{rno-miR-351-5p} \) may related to their channel regulation.

**Discussion**

The pathogenesis of DOP is complex. All the changes of function of osteogenesis and osteoclasts or structural performance of bone could lead to DOP. This research showed diabetes can result in BMD decreasing of long bone, which matched those from the previous reports\(^7\); studies like Abbassy showed, after 3-week old wistar group rats was induced diabetes for five weeks, their mandibular bone mineral density was severely decreased (Figure 2).

![Figure 1: Representative humerus microCT scan images of the three groups; the sham control group (A, D, G, J), the wistar group (B, E, H, K) and the diabetes group (C, F, I, L). Rats of diabetes group had less trabecular bone than wistar group and control group. Compared with control group, the BMD (bone mineral density) of humerus was significantly increased in wistar group and significantly reduced in diabetes group (M). Compared with wistar group, the humerus in diabetes group showed a significant reduction in BMD and BV/TV (bone volume relative to tissue volume), and a significant increase in Tb.Sp (trabecula spacing) (M, N, O, P). (*means \(P<0.05\) VS wistar; #means \(P<0.05\) VS control).](image)

![Figure 2: The differentially expressed miRNA scatter plot of sample wistar and sample diabetes. Each point in the figure represents a miRNA. The X axis and Y axis show expression level of miRNAs in two samples respectively. Red points represent miRNAs with fold-change>2. Blue points represent miRNAs with \(1/2<\text{fold-change}\leq2\). Green points represent miRNAs with \(\text{fold-change}\leq1/2\).](image)
down with increasing osteoclasts surrounded alveolar bone\(^8\).

Most selected differentially expressed miRNA in this study played a regulatory role in different diseases. In the different types of tumors, miR-10a can play a role of tumor suppressor\(^9\), also can play the role to promote cancer\(^10, 11\). Study showed miR-146a-5p has high expression in highly invasive osteosarcoma cell line, prompt its associated with the transfer ability of cancer cells\(^12\). miR-351 was associated with PC12 cell migration and differentiation\(^13\).

Some differentially expressed miRNA were associated with diabetes or the differentiation of mesenchymal stem cells (MSCs). Some studies indicated that whether people has diabetes or not, miR-199a-5p expression quantity of obese fat tissue were higher than that in normal people\(^14\).

After miR-199a-5p overexpression in human BMMSCs during adipogenic differentiation, the adipocyte marker gene FABP4 (aP2) expression decreased\(^15\). Another study found that miR-672-5p was associated with fat formation\(^16\). Knockout miR-146a-5p could inhibit the proliferation ability and enhance the migration ability of umbilical cord MSCs, while high expression of miR-146a-5p in the BMMSCs has no significant effect on the adipogenesis and osteogenetic differentiation ability\(^17\).

This study suggests that differentially expressed miRNA of DOP BMMSCs may relevant to Notch signal pathway. Notch signal pathway is an important and conservative signal transduction pathways influencing cell fate, almost involved in all cells proliferation and differentiation activity. In recent years, the function of Notch signal pathway to regulate the osteogenic differentiation of MSCs has gradually be taken seriously.

Notch signal has dual function of inhibition and inducing MSCs osteogenesis differentiation. Some research thinks Notch can promote MSCs osteogenesis differentiation. In-vivo study reported that the regulatory factor protein Rbpj in Notch signal pathway can promote the expression of MSCs osteogenesis differentiation markers such as Runx2\(^18\). Studies have also proved that Notch signal pathway associated with BMP2-Smad pathway through the target gene Hey1 and Hes1, can promote MSCs osteogenesis differentiation\(^19\).

Some people declaimed Notch can inhibit the MSCs osteogenetic differentiation, such as, under different promoter control, NICD respectively inhibited MSCs osteogenesis differentiation and osteoblast maturation according to mice experiments\(^20, 21\). Someone also found that Notch signal pathways can maintain undifferentiated state of MSCs cells, thus inhibiting bone-forming differentiation\(^22\). There are no reports about the miRNA affecting the differentiation of MSCs osteogenesis by Notch signal pathway.

This research use miRNA deep sequencing technology to filter differently expressed miRNA between normal rats and DOP rats, provide new ideas about further discusses of miRNA influences in diabetic osteoporosis.

References


Acknowledgments

This work was supported by the Natural Science Foundation of China (81271180 and 309973354).

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