Using Transgenic Mice to Explore the Effect of il-17rc Gene Knockout on Bone Metabolism

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Research Article

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Abstract

Objective

To investigate whether interleukin-17 receptor C (il-17rc) gene knockout leads to systemic osteoporosis in transgenic mice.

Methods

The immunohistochemistry and micro computed tomography (micro-CT) were used to analyze the condition of vertebral cancellous bone in 3-month healthy female wild-type C57BL/6 mice (control group) and il-17rc gene knockout C57BL/6 mice (experimental group).

Results

Wild type C57BL/6 mice had higher bone density per unit volume (0.52 ± 0.12 vs. 0.47 ± 0.05 , $P = 0.028$) (g/cm^3), more trabecular connections (8.97 ± 1.46 vs. 5.59 ± 3.15 , $P = 0.017$) (1/mm), thicker trabecular thickness (0.16 ± 0.08 vs. 0.10 ± 0.04 , $P = 0.029$) (1/mm) and the number of trabeculae was more (5.01 ± 0.33 vs. 3.16 ± 0.37 , $P = 0.038$) (1/mm) than mut-il-17rc mice. In addition, the results of micro-CT also showed that the osteoporosis of il-17rc gene knockout C57BL/6 mice was mainly manifested in T13 ($P = 0.039$), L1 ($P = 0.035$), L3 ($P = 0.018$), L5 ($P = 0.021$) and L6 ($P = 0.036$), but the mean bone mineral density between L2 ($P = 0.317$) and L4 ($P = 0.242$) was no significant difference between the two groups.

Conclusion

IL-17/il-17rs signal axis is widely distributed in the animal skeletal system and is one of the important signal pathways regulating bone metabolism. Knockout of il-17rc gene can lead to the occurrence of osteoporosis in model animals.

Background

Osteoporosis (OP) is a systemic multisystem bone disease, which is mainly characterized by decreased trabecular bone, collapse, decreased bone mineral density and increased bone fragility^[1]. Many clinical studies have found that abnormal immune system can lead to the occurrence and development of human osteoporosis^[2]. The previous genetic research of this subject found that the mutation of interleukin-17 receptor C (il-17rc) gene rs708567 (mut-il-17rc-rs708567) was significantly correlated with the occurrence and development of osteoporosis in Adolescent Idio-pathic Scoliosis (AIS) patients^[4,5]; This finding suggests that il-17rc gene mutation may be one of the causes of promoting the occurrence and development of osteoporosis. Il-17rc gene is in the region of chromosome 3p25.3-3.24.1, and rs708567 locus is in the exon region of il-17rc gene^[6]. DNA sequencing showed that rs708567 mutation in AIS patients could lead to the conversion of serine (SER, S) encoded by this site to leucine (Leu, L), forming a missense mutation from Ser to Leu. However, the above clinical research results have not been

confirmed by specific experiments. Therefore, the purpose of this study was to use transgenic mice to verify the effect of il-17rc gene mutation on the occurrence and development of osteoporosis.

Materials And Methods

Experimental animals and groups

A total of 20 healthy female C57BL/6 mice aged 3 months were included in this study, including 10 wildtype C57BL/6 mice as the control group and 10 il-17rc gene knockout C57BL/6 mice as the experimental group.

Experimental method

The expression of il-17rc gene in bone and muscle of wild-type C57BL / 6 mice was detected by immunohistochemistry. Reagent: Rabbit anti IL 17rc polyclonal antibody (bs-2607r, BIOSs), Rabbit Anti-IL 17rc Polyclonal Antibody (bs-1183R, Bioss), Goat anti rabbit IgG (H+L) (SA00004-2, Proteintech), DAB chromogenic Kit (cw0125, cwbio), Neutral balsam (cw0136, cwbio), Hematoxylin (H8070, Solarbio), etc.

Preparation of paraffin section pretreatment: Put the tissue slices into the oven at 65 ° C for 2h. Place the slices in xylene for 10 min and replace xylene for another 10 min. Put the slices into 100% ethanol, 100% ethanol, 95% ethanol, 80% ethanol and purified water for 5 minutes respectively.

Antigen retrieval: Put the slices into the antigen retrieval box, add antigen retrieval solution (citric acid buffer), heat the pressure cooker to automatically deflate, leave the heat source for natural cooling after 2 min, discard the antigen repair solution, and wash the slices with Phosphate-buffered saline (PBS).

Elimination of endogenous peroxidase: Move the slices into the wet box, add freshly prepared 3% hydrogen peroxide to remove the endogenous peroxidase blocking solution, incubate at room temperature for 10 min, and fully elute with PBS.

Blocking: The slides were soaked with PBS for 3 times, each time for 5 min. the PBS around the tissue was dried with absorbent paper, 5% BSA was added dropwise on the slides, and blocking at 37 ° C for 30 min.

Immunological reaction: Absorb the sealing solution around the tissue with absorbent paper without washing. Add sufficient diluted primary antibody: IL-17 (1:400) to each slide, put it into a wet box and incubate overnight at 4 °C. The next day, take out the wet box, let it stand at room temperature for 45 min, soak the slides with PBS for 3 times for 5 min each time, drop horseradish enzyme labeled Goat anti rabbit IgG (H+L) secondary antibody working solution (1:100), incubate at 37 ° C for 30 min, and rinse thoroughly with PBS.

Chromogen and counterstaining: The sections were washed three times (each time for 5 min) using PBS before chromogen was carried out with chromogenic reagents. Subsequently, counterstaining was

conducted with hematoxylin prior to dehydration, permeability, and mounting. Afterwards, sections were observed under a fluorescence microscope.

Preparation and identification of transgenic mice

The transgenic mice used in this experiment were produced by GemPharmatech company using wild-type C57BL/6 mice. The design scheme of transgenic mice is shown in Table 1.

Vector construction: According to the design scheme, the gRNA vector was designed and constructed, and the correctness of the vector sequence was verified by sequencing. gRNA was transcribed and purified in vitro.

Microinjection: Cas9 and gRNA samples were microinjected into mouse fertilized ovum with C57BL/6JGpt. The fertilized ovum survived after injection were transplanted into the pseudo pregnant female mice, and they were pregnant and gave birth.

Identification of F0 generation mice: The F0 generation mice born from the recipient mice were numbered by tail cutting and toe cutting in 5-7 days, and the genomic DNA was extracted for PCR and sequencing identification to confirm the genotype.

Breeding of positive F0 generation mice: After sexual maturation, F0 positive mice mated with wild-type background mice; The tail and toe of F1 mice were cut off in 5-7 days, and the genomic DNA was extracted for PCR and sequencing identification to confirm the genotype. (Figure 1)

Micro-CT detection: Mouse spine and femur specimens were placed in micro-CT and scanned along the long axis of the spine. The scanning voltage was 50 kV, the scanning current was 200 UA, and the pixel size was 9 $\mu\text{m} \times 9 \mu\text{m}$, layer spacing: 9 μm , the scanned image is imported into the 3D reconstruction software mimici0.0 for 3D reconstruction and relevant parameters are evaluated. Parameters includes the number of trabeculae (TB. N), trabecular space (TB. SP), trabecular thickness (TB. Th) and cortical thickness, etc.

Statistical analysis: SPSS 20.0 software is used for data processing, and the measurement data are expressed in ($\bar{x} \pm s$) indicates by t-test and $P < 0.05$ is statistically significant.

Results

In order to explore the expression of il-17rc gene in the bone and muscle tissue of wild-type C57BL/6 mice, this study used immunohistochemical staining to detect the expression of il-17rc protein in the bone and muscle tissue of C57BL/6 mice; the results showed that il-17rc protein is significantly expressed in the vertebral body, annulus fibrosus, femur, and paraspinal striated muscle of wild-type C57BL/6 mice; but il-17rc protein is expressed in the nucleus pulposus and femoral metaphyseal cartilage obviously decrease (Figure 2). The preparation and identification results of transgenic mice are shown in Table 2. In the identification results of F1 generation mice, according to the schematic diagram

of PCR strategy, confirmed by PCR and sequencing, 41[#]-44[#]48[#]-12bp, -3142bp/wt E2 - E6 were completely deleted, and the il-17rc gene was knocked out. 41[#] - 44[#]48[#] mice of F1 generation are positive results, detailed information is shown in Figure 3.

The bone mineral density of transgenic mice and wild-type C57BL/6 mice was detected by micro-CT. The test results indicate that compared with wild-type C57BL/6 mice, the average number of trabecular connections (5.01 ± 0.33 vs. 3.16 ± 0.37 , $P = 0.038$) (1/mm), thickness of trabecular bone (0.16 ± 0.08 vs. 0.10 ± 0.04 , $P = 0.029$) (1/mm), number of trabecular bones (8.97 ± 1.46 vs. 5.59 ± 3.15 , $P = 0.017$) (1/mm), and bone density (0.52 ± 0.12 vs. 0.47 ± 0.05 , $P = 0.028$) (g/cm³) of T13-L6 vertebral bodies of il-17rc gene KO mice are less. However, there was no statistically significant difference in the separation of trabecular bones of the cancellous bones of the vertebral body of the two groups of mice. (Table 3 and Figure 2)

Further analysis of the bone density of the cancellous bone of the T13-L6 vertebral body, the micro-CT test results indicate that in the il-17rc gene KO mice, osteoporosis is mainly manifested in the T13 ($P = 0.039$), L1 ($P = 0.035$), L3 ($P = 0.018$), L5 ($P = 0.021$) and L6 ($P = 0.036$) vertebrae. The average bone mineral density of L2 ($P = 0.317$) and L4 ($P = 0.242$) vertebrae was not significantly different between the two groups of mice. (Table 4)

Discussion

Interleukin-17 (iL-17) is a characteristic cytokine secreted by CD4⁺ cell subset Th17 cells, which plays an important role in the occurrence of inflammatory diseases and the regulation of bone metabolism^[7]. The transmission of IL-17 function depends on Interleukin-17/IL-17 receptor complex (IL-17/IL-17 receptors, IL-17/IL-17Rs) signal axis. The IL-17 receptor complex (IL-17Rs) family includes IL-17RA and there are 5 subunits including IL-17RB, IL-17RC, IL-17RD and IL-17RE^[8, 9]. IL-17RC is one of the important subunits of the IL-17 / IL-17 receptors signal axis complex One^[10, 11].

In this study, the researchers used IL-17RC gene knockout mice as experimental subjects and used micro-CT to detect the vertebral bone mineral density of IL-17RC gene knockout mice. The test results suggest that compared with wild-type C57BL/6 mice, the average number of trabecular connections, trabecular bone thickness, trabecular bone number, and bone density of T13-L6 vertebrae of il-17rc gene KO mice were less. However, there was no statistically significant difference in the trabecular separation of the cancellous bone per unit face of the two groups of mice. Based on the above results, the researchers believe that IL-17RC gene knockout can induce the occurrence of osteoporosis in mice.

Previous studies have found that excessive activation of the IL-17/IL-17R signal axis complex is one of the main causes of bone erosion in patients with rheumatoid^[12, 13].

Animal model studies have found that excessive activation of the IL-17/IL-17R signal axis can lead to osteoporosis in model animals^[14], while blocking the IL-17/IL-17R signal axis complex can significantly

reduce the degree of osteoporosis in animal models^[15]. In addition, in vitro studies have found that the IL-17/IL-17R signal axis complex can directly participate in the regulation of the expression of the nuclear factor kappa B activator 1 (ACT1) in osteoblasts. ACT1 is the main activation protein of the NF-κB receptor activator ligand / nuclear factor KB receptor activator 1 Receptor activator of NF-κB ligand, RANKL signaling pathway^[16]. As we all know, the RANKL/RANK signal axis is one of the important signal pathways for the regulation of bone metabolism^[17,18]. Although the specific role of IL-17RC subunits in IL-17Rs is still unclear, the results of this study suggest that after the IL-17RC gene is knocked out, the function of the IL-17/IL-17R signal axis complex may be activated, thereby promoting the occurrence of osteoporosis.

In this study, immunohistochemical technology was used to detect the location of il-17rc protein expression in wild-type C57BL/6 mice. The test results indicate that il-17rc protein is obvious expression in the intervertebral disc fibrous annulus, vertebral body, femoral cortical bone and cancellous bone, and paravertebral striated muscle. However, the expression in the intervertebral disc nucleus pulposus and femoral metaphysis cartilage was significantly decreased. The above test results suggest that the disease caused by the abnormal IL-17/IL-17R signal axis complex in the extensive division of il-17rc protein and the bone and striated muscle tissue in the body is also a systemic disease. When the micro-CT three-dimensional imaging technology was used to reconstruct the cancellous bone morphology of the T13-L6 vertebral body, the test results indicated that: in the T13-L6 vertebral body of the il-17rc gene KO mouse, osteoporosis is mainly manifested in T13, L1, L3, L5, L6 vertebral bodies, the average bone density of L2 and L4 vertebrae was not significantly different between the two groups of mice. Although most vertebral bodies have osteoporosis (T13, L1, L3, L5, L6 vertebral bodies), there are still some vertebral bodies without osteoporosis (L2, L4 vertebral bodies). There seems to be a certain contradiction between the test results of immune tissue and the test results of micro-CT. The researcher believes that in the later stage, it is necessary to increase the sample size of the experimental group of mice and the control group of mice to further explain the above contradiction.

Conclusion

This study uses immunohistochemical technology and micro-CT detection technology to confirm that IL-17/IL-17Rs signal axis is widely distributed in the skeletal system of animals and is one of the important signal pathways that regulate bone metabolism. Knockout of IL-17Rc gene can lead to the occurrence of osteoporosis in model animals. However, this study still has shortcomings. In the later study, we hope to correct the shortcomings of this study by increasing the sample size of the research object and expanding the detection range of specimens.

Abbreviations

il-17rc: interleukin-17 receptor C; KO: knockout; Micro-CT: micro computed tomography; OP: Osteoporosis; AIS: Adolescent Idiopathic Scoliosis; SER: serine; Leu: leucine; PBS: Phosphate-buffered saline; RANKL: Receptor activator of NF-κB ligand.

Declarations

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Medical research ethics committee of the First Affiliated Hospital of Nanchang University.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The authors have contributed in the following ways: Song Zhou was the lead author of the original report. Fan Yu provided concept/research design, data collection, data analysis, and manuscript writing. Bo Li and Guoliang Zhang provided concept/research design, analysis, and manuscript writing. All authors conceived of the study, took active part in all aspects of the study and read and approved the final manuscript.

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Not applicable.

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Tables

Table 1 Basic information of genes

Knockout gene name (NCBI number)	il-17rc171095
Knockout gene NCBI website	https://www.ncbi.nlm.nih.gov/gene/171095
Knockout gene name (MGI number)	il-17rcMGI :2159336
Knockout gene Ensembl website	http://asia.ensembl.org/Mus_musculus/Gene/Summary?g=ENSMUSG00000030281;r=6:113471427-113483140
Targeted transcript (Ensembl number)	il-17rc-201(ENSMUST00000058300.13)
Knockout gene exon	Exon 2-6

Table 2 Identification experiment results of F0 generation mice

gRNA sequence information

gRNA name	gRNA sequence	PAM
gRNA1	5'-CTAGGGGGCTGTCAAGCA-3'	AGG
gRNA3	5'-CACTTTGCCCGACACGA-3'	TGG

PCR primer information

PCR number	Primer name		Primer sequence	Product
KO	F1	GJS0219071124-01-il-17rc-KO-tF1	5'-GAAACCCTGTG GTCGTCTCTCTG-3'	WT:3821bp Targeted:~633bp
	R1	GJS0219071124-01- il-17rc-KO-tR1	5'-CTGAGTGACAAG AGGAGATGCCTG-3'	
WT	F2	GJS0219071124-01- il-17rc-wt-tF1	5'-GTAGGCAGCAAC TCAGATAGCCTG-3'	WT: 424bp Targeted:0bp
	R2	GJS0219071124-01- il-17rc-wt-tR1	5'-ATGGGGAGTGAC CAAAGGGACTG-3'	

Table 3 Comparison of the state of cancellous bone in T13-L6 vertebra

Measurement parameters	Control group	experimental group	<i>P</i>
Number of trabecular bone connections/mm	8.97 ± 1.46	5.59 ± 3.15	0.017
Trabecular bone thickness/μm	0.16 ± 0.08	0.10 ± 0.04	0.029
Number of trabeculae/mm	5.01 ± 0.33	3.16 ± 0.37	0.038
Trabecular bone separation/μm	0.24 ± 0.03	0.27 ± 0.02	0.087
Bone density/g/cm ³	0.52 ± 0.12	0.47 ± 0.05	0.028

Table 4 Comparison of cancellous bone mineral density of T13-L6 vertebral body

Vertebral body	Control group g/cm ³	experimental group g/cm ³	<i>P</i>
T13	0.51±0.13	0.46±0.07	0.039
L1	0.53±0.16	0.45±0.13	0.035
L2	0.45±0.09	0.45±0.16	0.317
L3	0.56±0.14	0.42±0.11	0.018
L4	0.49±0.11	0.48±0.21	0.242
L5	0.54±0.19	0.48±0.09	0.021
L6	0.52±0.26	0.42±0.12	0.036

Figures

Figure 1

Identification results of F1 generation mice. ☐ PCR reaction did not get Target band; ☐ PCR reaction can get WT band. Heterozygous: ☐ PCR reaction can get Target band; ☐ PCR reaction can get WT band. Homozygous: ☐ PCR reaction can get Target band; ☐ PCR reaction does not get WT band.

Figure 2

Results of immunohistochemical staining of different tissues. Brown is il-17rc protein positive staining. Figure 2A: A single intervertebral disc and upper and lower vertebral body tissues. Il-17rc protein is expressed in the annulus fibrosus and upper and lower vertebral bodies, but low expression in the nucleus pulposus tissue of the intervertebral disc. Figure 2B: It is the bone tissue of the distal femur. Il-17rc protein is expressed in femoral cortical bone and cancellous bone tissue, but low expression in femoral

metaphyseal cartilage. Figure 2C: It is the paravertebral striated muscle tissue. The il-17rc protein is obviously expressed in the paravertebral striated muscle.

Figure 3

Results of electrophoresis. The numbers are mouse tail numbers, WT: wild type, N: negative blank control, and M: DNA Marker.

Figure 4

Micro-CT three-dimensional reconstruction images of cancellous bone of wild-type and transgenic mice T13-L6 vertebral body. A: wild-type C57BL/6 mice. Figure 4B: il-17rc knockout mice; wild-type C57BL/6

mice have more trabecular bone connections per unit volume (1/mm) and thicker trabecular bone thickness (um) The number of bone trabeculae (1/mm) is more, compared with mut-il-17rc mice.