Distinctive Expression of Bone Metabolism-related Genes between PBMCs from Condylar Hyperplasia, Rheumatoid Arthritis, and Ankylosing Spondylitis Patients

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ABSTRACT

Bone morphogenetic proteins (BMPs) and wingless (Wnt) signaling molecules and their antagonists, such as sclerostin and noggin, have been identified to have different effects on bone metabolism. This research intended to evaluate the transcript levels of CTNNB1 (catenin beta 1 protein), SOST (sclerostin protein), BMP4 (Bone Morphogenetic Protein 4 protein), and NOG (noggin protein) bone metabolism-related genes in peripheral blood mononuclear cells (PBMCs) from condylar hyperplasia (CH) patients in comparison to rheumatoid arthritis (RA), ankylosing spondylitis (AS), and healthy individuals.

PBMCs were separated from blood samples of 10 patients with CH, AS, RA, and 10 healthy controls. SYBR Green real-time polymerase chain reaction (PCR) was used for quantitative analysis of CTNNB1, SOST, BMP4, and NOG messenger RNAs (mRNAs).

The expression of CTNNB1 was significantly upregulated in CH and AS patients compared with healthy individuals and RA patients. The difference of SOST expression was not significant between all groups. The BMP4 expression was significantly downregulated in AS, CH, and RA patients compared with healthy controls. The NOG expression was downregulated in RA, AS, and CH groups, however, it was only significant in CH and RA patients compared with controls. CH and AS patients were distinguished from RA by the upregulated CTNNB1 expression.

These results demonstrated that CTNNB1, BMP4, and NOG, but not SOST, may contribute to the pathogenesis of CH, AS, and RA.

Keywords: Ankylosing spondylitis; Bone morphogenetic proteins; Rheumatoid arthritis
INTRODUCTION

Various abnormal bone architectures observed in rheumatic disorders such as rheumatoid arthritis (RA) and ankylosing spondylitis (AS), which are induced when the balance between bone resorption and formation is broken. A rare rheumatic disease, condylar hyperplasia (CH), causes by abnormal growth in the mandible and affects the condylar neck and head. The main manifestations of CH are facial and mandibular asymmetry, chin, and dental midline deviation toward the opposite side.1

The bone morphogenetic protein (BMP) and canonical wingless (Wnt) are two bone promoting pathways that are essential for bone remodeling.2 During the canonical Wnt pathway, β-catenin, encoded by catenin beta 1 (CTNNB1) gene, is stabilized and translocated to the nucleus, leading to transcription of various genes, which controls the osteoblast differentiation. The dickkopf-related protein 1 encoded by the DKK gene and sclerostin, encoded by the SOST gene, are two types of the Wnt signaling antagonists.3 Noggin, encoded by the NOG gene, is enumerated as an antagonist of BMPs.4 Also, sclerostin is another BMPs antagonist, which is related to both BMPs and Wnt pathways.5

BMP-4 stimulated bone formation, whereas BMP-5, -6, and -8 are involved in chondrogenesis.6 In CH patients, BMP-2 was observed mainly in the proliferative and hypertrophic chondrocytes layer. The balance between BMPs and noggin is important in osteogenesis and overexpressed noggin in mice led to osteopenia and disrupted osteoblastic function.8

There is no significant comparative analysis of bone remodeling pathways in various types of rheumatic diseases, especially in CH. Accordingly, we intended to assess the mRNA expression of CTNNB1, SOST, BMP4, and NOG in CH, AS, RA, and healthy control groups.

MATERIALS AND METHODS

Study Participants

Participants in this study consisted of 4 groups of CH, AS, RA, and healthy subjects (n=10 each). The CH and AS/RA patients were recruited from the department of oral and maxillofacial surgery, and outpatient clinics of Rheumatology Research Center, Shariati Hospital, Tehran, Iran respectively. All groups were matched to each other according to sex (5 men and 5 women in each group) and age (25.4±8.20, 29±12.19, 34.6±9.26, and 28.3±6.78 years old in CH, AS, RA, and healthy groups, respectively). CH was confirmed by the clinical and radiographic evaluation that includes mandibular deviation and condylar head growth without any deformity and enlargement. Scintigraphy with a Tc99m scan (bone scan) shows cellular activity in the condylar region that is 10% differences between the left and right side. AS and RA patients fulfilled the modified New York 1984 criteria and American College of Rheumatology (ACR) criteria; respectively.7,10 Healthy donors without any evidence of rheumatologic or autoimmune disorders or facial asymmetry (chin, dental midline) in neither themselves nor immediate family members were randomly included in this study. This study was approved by the ethics committee at the Tehran University of Medical Sciences. (Approval ID: IR.TUMS.VCR.REC.1396.3779).

Sample Collection and RNA Isolation

PBMCs were purified by density gradient centrifugation method from 5 milliliters of whole blood samples from each subject; using Ficoll-Hypaque. Total RNA was separated from PBMCs by the High Pure RNA Isolation Kit (Roche, Germany), following the manufacturer’s instruction. The quality of the isolated RNA was checked; using the NanoDrop spectrophotometer apparatus (2000c, Thermo Fisher Scientific, USA) at 260/280 nm.

cDNA Synthesis and Real-time Quantitative PCR

One µg of isolated RNA was reverse-transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) following the manufacturer’s manual. The cDNA samples were stored at -20°C for later analysis. PCR primer sequences (supplementary file 1) were retrieved from the PCR Primer Bank database (https://pga.mgh.harvard.edu/primerbank/). The Basic Local Alignment Search Tool (BLAST) on the NCBI website was used to assess the quality and specificity of each primer. Quantitative PCR was carried out with the SYBR Green qPCR master mix (Takara Bio, Inc.) and Step One Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA). The expression data were analyzed by the comparative CT method, which previously explained by Livak and Schmittgen.11
β2-microglobulin (β2M) mRNA transcript level was considered as a housekeeping gene.

**Statistical Analysis**

Data were analyzed using SPSS software v.25 (SPSS, Chicago, IL, USA). The Mann-Whitney U test was exerted to compare groups. Data were plotted by the GraphPad Prism version 7.00 software (GraphPad Software, La Jolla, California, USA, www.graphpad.com). Scale data were expressed as mean ± standard error of the mean (SEM). Statistically, significance was considered at *p* value<0.05.

**RESULTS**

The mRNA Expression Level of the CTNNB1, SOST, BMP4, and NOG Gene

The mRNA expression of the CTNNB1 gene was upregulated in PBMCs from AS and CH patients by 1.55 and 1.42 fold respectively, compared to healthy control (*p*=0.03 and *p*=0.04, respectively) by 2.31 and 2.11 fold, in comparison to RA patients (*p*<0.001). In addition, PBMCs from RA patients expressed lower CTNNB1 level compared to healthy PBMCs (*p*=0.02, FC=0.67) (Figure 1A).

There were no significant differences in SOST expression between the studied groups (Figure 1B). The expression of the BMP4 gene in PBMCs of CH, AS, and RA patients were diminished by 0.15, 0.13, and 0.09 fold respectively in comparison to the healthy individuals (*p*=0.003, *p*<0.001, *p*<0.001). The expression of BMP4 did not show any significant difference between PBMCs from CH and AS or RA group (Figure 1C).

Figure 1. Bar graphs to illustrate the relative gene expression of CTNNB1 (A), SOST (B), BMP4 (C), and NOG (D) in PBMCs from CH, RA, and AS patients compared to healthy controls and each other. The mRNA expression level of CTNNB1 in CH and AS patients were significantly upregulated, in comparison to healthy controls and RA patients (A), there were no significant differences in the expression level of SOST between the studied groups (B), the expression level of BMP4 was significantly downregulated in CH, RA and AS compared with healthy controls (C), there was also a significant decreased in the expression of NOG between CH and RA compared to healthy controls (D). (AS; ankylosing spondylitis, RA; rheumatoid arthritis, CH; condylar hyperplasia, CO; control, PBMCs; peripheral blood mononuclear cells, *P*; *P*-values, FC; fold change).
BMP4 patients expressed NOG, with active bone formation, and downregulated in osteocytes led to osteopenia which is associated with and loss of β-catenin in mature osteoblasts and activation of β-catenin led to osteoblast differentiation healthy control. Earlier studies revealed that the RA with inadequate bone formation compared with radiographic joint damages in RA and AS, later studies have shown a relationship between sclerostin level and healthy groups. Although some previous studies of the wnt/β catenin pathway had no significant demonstrated no significant difference in sclerostin than in RA patients (p<0.001). We did not observe a difference between PBMCs from the control group and AS; ankylosing spondylitis, RA; rheumatoid arthritis, CH; condylar hyperplasia, HC; healthy control, p; p-values.

mRNA expression of NOG was subsided by 0.44 and 0.18 fold in PBMCs from CH and RA group respectively, compared to healthy controls (p=0.02 and p<0.001, respectively). We did not observe a significant difference in NOG expression between CH and AS or RA patients. Although, PBMCs from AS patients expressed NOG mRNA 3.85 fold higher than PBMCs from RA patients (p<0.001, Table 1 and Figure 1D).

The ratio of NOG to BMP4 had no significant difference between PBMCs from the control group and CH, RA, or AS patients. However, the ratio of NOG to BMP4 in PBMCs from AS patients was 3-fold higher than in RA patients (p<0.001).

DISCUSSION

In this study, CTNNB1 was upregulated in CH and AS, with active bone formation, and downregulated in RA with inadequate bone formation compared with healthy control. Earlier studies revealed that the activation of β-catenin led to osteoblast differentiation and loss of β-catenin in mature osteoblasts and osteocytes led to osteopenia which is associated with activated osteoclastogenesis. In our study, the expression of SOST as an inhibitor of the wnt/β catenin pathway had no significant difference within patient groups and between patients and healthy groups. Although some previous studies have shown a relationship between sclerostin level and radiographic joint damages in RA and AS, later studies demonstrated no significant difference in sclerostin level in RA and AS patients in comparison to the healthy group. Hence conflicting data have been reported, a meta-analysis on sclerostin suggested that this difference in results was related to the small size of the population under investigation and the presence of the intervening factors such as sex and age.

Here, BMP4 gene expression was remarkably downregulated in CH, AS and RA PBMCs compared to the healthy group. In line with our results, another study showed that BMP4 production in RA patients decreases in synovial tissues. However, a previous study has revealed that the BMP4 serum level in AS patients increased and play a role in the progress of the disease. Moreover, other studies demonstrated that suppression of BMP4 by noggin can lead to protection against AS and BMP4 plays the main role in the differentiation of skeletal progenitor cells to chondrocytes in the early stage of AS. There are no previous reports about BMP4 expression and function in CH patients.

We have also found decreased expression of NOG in CH and surprisingly RA compared with healthy controls. PBMCs from all studied groups express more NOG than BMP4 and the ratio of NOG to BMP4s is still in favor of inhibition of bone formation in RA PBMCs. Decreased noggin level in Nog^+/+;LacZ mice is a protective mechanism against articular cartilage damage in arthritis. It seems that a protective mechanism occurred in our RA group like Nog^+/+;LacZ mice. According to our results, NOG expression in PBMCs from AS patients was significantly elevated than in the RA group. On the other hand, the balance between

| Gene       | CH vs. HC Fold change(p) | AS vs. HC Fold change(p) | RA vs. HC Fold change(p) | CH vs. AS Fold change(p) | CH vs. RA Fold change(p) | AS vs. RA Fold change(p) |
|------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| CTNNB1     | 1.42                     | 1.55                     | 0.67                     | 9.83                     | 2.11                     | 2.31                     |
|            | (0.04**)                 | (0.03**)                 | (0.02**)                 | (0.47)                   | (< 0.001***)             | (< 0.001***)             |
| SOST       | 1.65                     | 0.17                     | 0.17                     | 9.89                     | 1.01                     |
|            | (0.41)                   | (0.31)                   | (0.11)                   | (0.39)                   | (0.63)                   |
| BMP4       | 0.15                     | 0.13                     | 0.09                     | 10.68                    | 14.93                    |
|            | (0.003***)               | (<0.001***)              | (<0.001***)              | (0.20)                   | (0.10)                   |
|            | (0.60)                   | (0.39)                   | (0.11)                   | (0.39)                   |
| NOG        | 0.44                     | 0.69                     | 0.18                     | 0.64                     |
|            | (0.02**)                 | (0.09)                   | (<0.001***)              | (0.15)                   |
|            | (0.02)                   | (0.09)                   | (<0.001***)              | (0.06)                   |
|            |                          |                          |                          |                          | 3.85                     |

AS; ankylosing spondylitis, RA; rheumatoid arthritis, CH; condylar hyperplasia, HC; healthy control, p; p-values.

*p values in the bold form are statistically significant (*p value< 0.05, **p value< 0.01, and ***p value< 0.001)
BMPs and NOG expressions have significant effects on bone formation at different stages of AS.20,22 We observed that the ratio of NOG to BMP4 in PBMCs from AS patients was 3-fold higher than in RA patients. This increase may reflect a protective mechanism against bone formation in AS patients.

Overall, we found that CH patients had significant upregulation of CTNNB1. On the other hand, NOG and BMP4 were downregulated in CH patients in comparison to healthy individuals. The CH and AS patients were significantly distinguished from RA patients by the upregulated CTNNB1 expression. None of the studied genes had significant differences between PBMCs from CH and AS patients. Unlike AS and RA, there is not much research on the pathogenesis of CH, therefore, CH needs more investigations regarding understanding the regulation of bone signaling pathways.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES

1. Yoshida H, Hayashi S-I, Kunisada T, Ogawa M, Nishikawa S, Okamura H, et al. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature. 1990;345(6274):442.
2. Regard JB, Zhong Z, Williams BO, Yang Y. Wnt signaling in bone development and disease: making stronger bone with Wnts. Cold Spring Harb Perspect Biol. 2012;4(12).
3. Monroe DG, McGee-Lawrence ME, Oursler MJ, Westendorf JJ. Update on Wnt signaling in bone cell biology and bone disease. Gene. 2012;492(1):1-18.
4. Chen G, Deng C, Li YP. TGF-β and BMP signaling in osteoblast differentiation and bone formation. Int J Biol Sci. 2012;8(2):272-288.
5. Wang SY, Liu YY, Ye H, Guo JP, Li R, Liu X, et al. Circulating Dickkopf-1 is correlated with bone erosion and inflammation in rheumatoid arthritis. J Rheumatol. 2011;38(5):821-827.
6. Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. Growth Factors. 2004;22(4):233-241.
7. MENG Q, LONG X, DENG M, CAI H, LI J. The expressions of IGF-1, BMP-2 and TGF-β1 in cartilage of condylar hyperplasia. Journal of Oral Rehabilitation. 2011;38(1):34-40.
8. Devlin RD, Du Z, Pereira RC, Kimble RB, Economides AN, Jorgetti V, et al. Skeletal overexpression of noggin results in osteopenia and reduced bone formation. Endocrinology. 2003;144(5):1972-1978.
9. Van der Linden S. Evaluation of diagnostic criteria for ankylosing spondylitis. Curr Opin Rheumatol. 1984;8:269-274.
10. Neogi T, Aletaha D, Silman AJ, Naden RL, Felson DT, Aggarwal R, et al. The 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for rheumatoid arthritis: Phase 2 methodological report. Arthritis Rheum. 2010;62(9):2582-2591.
11. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protocols. 2008;3(6):1101-1108.
12. Hartmann C. A Wnt canon orchestrating osteoblastogenesis. Trends Cell Biol. 2006;16(3):151-8.
13. Huang C, Wang JJ, Ma JH, Jin C, Yu Q, Zhang SX. Activation of the UPR protects against cigarette smoke-induced RPE apoptosis through up-regulation of Nrf2. J Biol Chem. 2015;290(9):5367-80.
14. Ustun N, Tok F, Kalyoncu U, Yuksel R, Yagiz A, Guler H, et al. Sclerostin and Dkk-1 in patients with ankylosing spondylitis. Acta reumatologica portuguesa. 2014; 39(2).
15. Shi J, Ying H, Du J, Shen B. Serum Sclerostin Levels in Patients with Ankylosing Spondylitis and Rheumatoid Arthritis: A Systematic Review and Meta-Analysis. Biomed Res Int. 2017;2017:9295313.
16. Ibrahim SE, Abdelsamad AM, Helmy A, Farouk N. Serum sclerostin levels in rheumatoid arthritis. Indian Journal of Rheumatology. 2015; 10(3):117-120.
17. Shi J, Ying H, Du J, Shen B. Serum Sclerostin Levels in Patients with Ankylosing Spondylitis and Rheumatoid Arthritis: A Systematic Review and Meta-Analysis. Biomed Res Int. 2017;2017:9295313.
18. Bramlage CP, Haupi T, Kaps C, Ungethum U, Krenn V, Pruss A, et al. Decrease in expression of bone morphogenetic proteins 4 and 5 in synovial tissue of
patients with osteoarthritis and rheumatoid arthritis. Arthritis Res Ther. 2006;8(3):R58.

19. Chen HA, Chen CH, Lin YJ, Chen PC, Chen WS, Lu CL, et al. Association of bone morphogenetic proteins with spinal fusion in ankylosing spondylitis. J Rheumatol. 2010;37(10):2126-2132.

20. Carter S, Braem K, Lories RJ. The role of bone morphogenetic proteins in ankylosing spondylitis. Ther Adv Musculoskelet Dis. 2012; 4(4):293-299.

21. Lories RJU, Luyten FP. Bone morphogenetic proteins in destructive and remodeling arthritis. Arthritis Res Ther. 2007;9(2):207-207.

22. Xie Z, Wang P, Li Y, Deng W, Zhang X, Su H, et al. Imbalance Between Bone Morphogenetic Protein 2 and Noggin Induces Abnormal Osteogenic Differentiation of Mesenchymal Stem Cells in Ankylosing Spondylitis. Arthritis Rheumatol. 2016;68(2):430-440.