The effects of Neuroblastoma Breakpoint Family Members 1 and 15 variant on osteogenesis in patients with orbital hypertelorism

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Research article

Keywords: orbital hypertelorism, NBPF1, NBPF15, osteogenesis

Posted Date: December 10th, 2019

DOI: <https://doi.org/10.21203/rs.2.18399/v1>

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Abstract

Background: The purpose of this study was to examine the effects of Neuroblastoma Breakpoint Family, Member 1, (NBPF1) and Neuroblastoma Breakpoint Family, Member 15, (NBPF15) on osteogenesis in patients with orbital hypertelorism.

Methods: The genetic information of three patients with orbital hypertelorism was analyzed via gene sequencing. We used real-time PCR to determine osteogenic correlation indices and performed osteogenic correlation staining to examine the function of NBPF1 and NBPF15 in the state of silence and overexpression.

Results: NBPF1 and NBPF15 gene variants were observed in patients with orbital hypertelorism. During osteogenesis, when NBPF1 and NBPF15 were overexpressed, real-time PCR showed that expression of the osteogenesis-related indicators alkaline phosphatase (ALP), runt-related transcription factor 2, osteopontin, and collagen type I alpha 1 chain increased; Alizarin Red and ALP staining showed that overexpression of both genes promoted osteogenesis. When NBPF1 and NBPF15 were silenced, expression of ALP, runt-related transcription factor 2, osteopontin, and collagen type I alpha 1 chain decreased as observed during real-time PCR, and the inhibition of osteogenesis was demonstrated by Alizarin Red and ALP staining.

Conclusion: NBPF1 and NBPF15 variants existed in patients with orbital hypertelorism and they promoted osteogenesis.

Background

Orbital hypertelorism was proposed and named by Greig in 1924[1]. It refers to severe craniofacial deformity involving excessive widening between the eyelids on both sides. It is a clinical symptom and can occur in many types of craniofacial deformities such as Tessier craniofacial fractures, 0–14 craniofacial fissure, Apert syndrome, and Crouzon syndrome[2]. Long-distance syndrome is characterized by excessive sacral frontal stenosis, frontotemporal dysplasia, cleft palate, or syndromic craniosynostosis[3, 4]. However, the pathogenesis of the disease remains unclear. Tessier[3] attributed true over-range to the causes of pathogenesis and pathological heterogeneity including (1) frontal sinus malformation, (2) frontal sinus dysplasia, (3) craniofacial lateral fissure, and (4) brain swelling.

DNA sequencing is a practical technology based on the enzymology and biochemistry of nucleic acids. It has been used widely in basic research, molecular biology, and the study of gene structure and function, and it can be used to analyze patients' genetic information.

The Neuroblastoma Breakpoint Family (NBPF) gene has a repeating structure in both the coding region and the noncoding region known as the domain of unknown function (DUF) 1220 and has high intra- and inter-gene sequence similarity. These similarities could cause unreasonable recombination of these genomic regions, resulting in structural variation of the NBPF gene. Further, this gene family shows

primate-specific duplication, reflecting the continual evolution of primate genomes and leading to considerable physiological differences[5]. Previous studies have shown that the number of copies of this region in human brain evolution is related to brain size[6–9], certain brain diseases (autism/schizophrenia/microcephaly and macrocephaly), and cognitive function[7, 10–12]. In addition, some studies have shown a robust linear association between NBPF/DUF1220 copy number and primate brain size. Therefore, this study aimed to determine whether there was a correlation between NBPF genes and bone formation. It is the first study to examine the NBPF Member 1 (NBPF1) and Member 15 (NBPF15) genes in the osteogenesis process.

Methods

Patient sample

Blood samples were drawn from two patients for gene sequencing.

Cell lines and reagents

The human bone marrow-derived mesenchymal stem cells (hBMSCs) used in the experiment were obtained from the stem cell bank at the Chinese Academy of Sciences (No. SCSP-405). The hBMSC cells were maintained in Dulbecco's Modified Eagle Medium (Gibco, Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) in a cell incubator (37 °C, 5% CO₂).

The osteogenic induction fluid formula was as follows: 10% fetal bovine serum Dulbecco's Modified Eagle Medium (low glucose), 1×10^{-8} mol/L dexamethasone, ascorbic acid 2×10^{-4} mol/L, and beta-phosphate glycerol 10 mM/L.

Real-time PCR

The hBMSCs were osteogenically induced for 0, 7, 14, and 21 days. Total RNA was extracted using the TRIzol method; reverse transcription was performed using Prime Script™ RT Master Mix (Perfect Real Time; Takara, Otsu, Japan), and gene expression was detected via TB Green™ Premix Ex Taq™ (Takara) following manufacturer's protocols. The primers used in the experiment were all synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

Plasmid construction and cell transfection

The NBPF1 and NBPF15 plasmids used in the experiment were constructed by Hanyin Biotechnology (Shanghai) Co., Ltd. (Shanghai, China) using the following steps. The synthetic primers were designed to amplify the target fragment and ligated into the enzyme through the restriction sites at both ends. After excision overexpressing the lentiviral vector, the ligation product was transferred into the prepared bacterial competent cells, and the grown monoclonal colonies were identified by PCR. The positive colonies were identified by PCR and used for sequencing and identification by comparing with the correct clones. A successful target gene was constructed to overexpress a lentiviral vector. The vector used was

CMV-MCS-3XFlag-PGK-Puro, restriction site 1: XhoI, restriction site 2: EcoRI. This vector was transfected into hBMSCs using the virus solution prepared by the company, with polybrene as a transfection reagent, and the expression levels of NBPF1 and NBPF15 were detected using real-time PCR (Fig. 1a). The constructed overexpressing cells were used in the experiment, and the control group was transfected with empty plasmid. Gene expression of the osteogenic indicators alkaline phosphatase (ALP), runt-related transcription factor (RUNX2), collagen type I alpha 1 chain (COL1a), and osteopontin (OPN) was detected via real-time PCR (Fig. 2).

Small interfering RNA construction and cell transfection

The NBPF1 and NBPF15 small interfering RNAs (siRNAs) were synthesized by Guangzhou Ruibo Biotechnology Co., Ltd. (Guangdong, China) and used to transfect hBMSCs to silence NBPF1 and NBPF15 genes. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used as a transfection reagent. The specific steps were as follows: When the cells were fused at 70–80% cell confluency, the siRNA was dissolved in 50 μ L of Opti-mem serum-free medium. In addition, 2 μ L of Lipofectamine 2000 was dissolved in another tube with 50 μ L of Opti-mem serum-free medium, mixed, and allowed to stand at room temperature for 5 min. The two tubes were mixed and left to stand for 20 min. The medium was changed to serum-free medium during transfection, and the mixture was added to the corresponding wells (the final concentration of siRNA was 100 nm). After 6 hours, the medium was replaced with serum medium. Total RNA was extracted at 48 hours, and siRNA silencing efficiency was detected by real-time PCR (Fig. 1b). The siRNA with the best silencing effect was used in subsequent experiments. The control group was transfected with a negative control provided by the company. Osteoblasts were induced for 0, 7, 14, and 21 days. Real-time quantitative polymerase chain reaction was used to detect the expression of the osteogenic indicators ALP, RUNX2, and OPN (Fig. 3). AR staining and ALP staining results counted using Image J[13].

Statistical analysis

Whole-exome sequencing data analysis

The sequencing readings generated by the Hiseq series were mapped to the human genome (hg19) using BWA software [14]. The resulting bam files were then imported to VarScan2 software 9[15], and germline variants were called using the following parameters: min-coverage: 20 min-var-freq: 0.08, and p value: 0.05. The fpfilter module of VARSCAN was applied to identify false positive variations. SnpEff and Variant Effect Predictors 10 and 11 were used to annotate the filtered variants. Further, we applied the following criteria to filter germline variants: (1) depth of alternative alleles: >5; (2) allele frequency of the alternative allele in the population: <0.001; (3) variant ratio of the alternative allele: >0.3; (4) impact severity = “HIGH” or “MED”; and (5) the prediction result of POLEPHEN2 was not “benign,” and the prediction result of SIFT was not “tolerated.”

Real-time PCR

The real-time PCR data were analyzed using the $2^{-\Delta\Delta C_T}$ method normalized to the mean C_t values of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase. Two-fold changes in gene expression (both decreases and increases) were considered statistically significant. The ΔC_t value for the control group was set at 1. The expression level of each indicator gene was calculated.

Staining statistics

The image was counted stained area using Image J. Three fields were selected for each well to calculate the average value. Three replicate wells were used in the statistical analysis; $P < 0.05$ was considered statistically significant.

Results

Gene sequencing

The results of genetic analysis showed NBPF gene variants in patients with orbital hypertelorism. One patient showed NBPF1 site variants (rs200611498 located in Chr 1), and the other showed NBPF15 variants at the same site (rs200292005 located in DUF1220) (Table 1).

Expression of NBPF1 and NBPF15 during osteogenesis

The results showed that the expression levels of NBPF1 and NBPF15 changed during osteogenesis (Fig. 1c).

Effect of overexpression of NBPF1 and NBPF15 on osteogenesis

ALP staining at 7 days of induction and Alizarin Red (AR) staining at 21 days (Fig. 4) indicated that NBPF1 and NBPF15 promoted osteogenesis when overexpressed. Observations under the microscope showed that the overexpression group was promoted in ALP and AR staining. The results showed a statistical difference (Fig. 5a).

Effect of silencing NBPF1 and NBPF15 on osteogenesis

ALP staining at 7 days of induction and AR staining at 21 days (Fig. 6) showed inhibition of osteogenesis by NBPF1 and NBPF15. Under the microscope, the silenced group was inhibited in ALP and AR staining. The results showed a statistical difference (Fig. 5b).

Discussion

Orbital hypertelorism, which is commonly treated in plastic surgery, is found in a variety of craniofacial deformities. At present, the pathogenesis of the widening of the gap remains ambiguous, and no clear conclusion has been made via the exploration of genes.

We know that the NBPF gene plays a role in the evolution of primates, including humans[16], and the DUF1220 protein domain in the NBPF gene has the most extreme human lineage specificity in all protein coding regions of the human genome[16, 17]. Areas with increased copy numbers have recently been associated with evolutionary and pathological changes in brain size (e.g., 1q21-related microcephaly)[18]. These findings support the notion that the DUF1220 regional dose is a key factor in determining brain size in primates (including humans). NBPF1 was first identified in patients with neuroblastoma because of disruption of a restarted structural translocation between 1p36.2 and 17q11.2 chromosomes [19, 20]. There is increasing evidence that changes in NBPF1 expression are associated with various cancer pathogenesises including gastric cancer and neuroblastoma[21]. In addition, a recent article reported that NBPF1 exerted a suppressive effect on tumors by inducing G1 cell cycle arrest in neuroblastoma[22]. Moreover, gene variants in NBPF1 and NBPF15 have been found in skull base chordoma[23].

The current gene sequencing results showed that patients with orbital distention exhibited NBPF1 and NBPF15 gene variants. According to existing reports, the NBPF has been observed in some patients with microcephaly or malformation. Variants were observed in family-related genes; therefore, NBPF1 and NBPF15 are highly likely to affect osteogenesis. In the current experiments, the expression of NBPF1 and NBPF15 increased significantly during the induction of osteogenesis via hBMSCs. This finding also suggests that NBPF1 and NBPF15 could participate in the osteogenesis process. NBPF1 and NBPF15 were overexpressed in hBMSCs through the overexpression of plasmids, and osteogenic induction was performed. Following osteogenic induction for 7, 14, 21 days, the osteogenic indicators ALP, RUNX2, OPN, and COL1A increased significantly with NBPF1 and NBPF15 overexpression. ALP staining was performed at 7 days of osteogenic induction, and AR staining was performed at 21 days of osteogenic induction. The staining showed that NBPF1 and NBPF15 overexpression was significantly greater in the experimental group relative to that of the control group. The above results indicate that NBPF1 and NBPF15 promoted osteogenesis when overexpressed.

In addition, siRNAs of NBPF1 and NBPF15 were used to transfect hBMSCs, and osteogenic induction was performed. Real-time PCR showed osteogenesis-related indicators at 7, 14, and 21 days of osteogenesis. When NBPF1 and NBPF15 were silenced, the expression of the osteogenesis-related indicators ALP, RUNX2, OPN, and COL1A decreased significantly. ALP and AR staining were performed at 7 and 21 days of osteogenic induction, respectively, and when NBPF1 and NBPF15 were silenced, the staining observed in the experimental groups was worse relative to that observed in the control group. These results indicate that NBPF1 and NBPF15 inhibited osteogenesis when silent.

Conclusion

The results showed variants in NBPF1 and NBPF15 in patients with orbital hypertelorism. Specifically, osteogenesis-related experiments demonstrated that NBPF1 and NBPF15 promoted osteogenesis. Therefore, variants in NBPF1 and NBPF15 are likely to affect the performance of osteogenesis, leading to a widening of the interpupillary distance. The specific mechanism underlying this requires further exploration.

Declarations

Ethics approval and consent to participate

Two patients with clinically diagnosed orbital distention syndrome or their guardians provided informed consent to participate in the study. The study was approved by the scientific research ethics committee at Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine.

Consent for publication

Written informed consent was obtained from all patients included in this report for the use of clinical-related materials for scientific research and publications.

Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due Some of the information involved patients' genes and we need to protect the patient's genetic information but are available from the corresponding author on reasonable request.

Competing interests

None

Funding

This work was supported by the Emergency management project of National Natural Science Foundation of China (grant number 81741012)

Authors' contributions

N H conducted experiments NH and QZ wrote manuscripts; T L and W C conducted statistical guidance and writing guidance; Y Z and G C were the project supervisors, guiding and coordinating the project. All authors read and approved the final manuscript

Acknowledgements

We would like to thank Editage (www.editage.cn) for English language editing.

Abbreviations

ALP, alkaline phosphatase; AR, Alizarin Red; COL1a, collagen type I alpha 1 chain; DUF, domain of unknown function; hBMSC, human bone marrow-derived mesenchymal stem cell; NBPF1, Neuroblastoma Breakpoint Family, Member 1; NBPF15, Neuroblastoma Breakpoint Family, Member 15; OPN, osteopontin; RUNX2, runt-related transcription factor; siRNA, small interfering RNA.

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Table

Due to technical limitations, Table 1 is only available as a download in the supplemental files section