

Difference in osteogenic ability of bone marrow mesenchymal stem cells in patients with hemifacial microsomia on the healthy side versus affected side of the mandible

nan huang

Shanghai Jiao Tong University School of Medicine <https://orcid.org/0000-0002-0862-4948>

Tianya Li

Shanghai Jiao Tong University School of Medicine

Wei Chen

Shanghai Jiao Tong University School of Medicine

Qun ZHANG

Shanghai Jiao Tong University School of Medicine

Gang CHAI (✉ chaig1081@sh9hospital.org)

<https://orcid.org/0000-0003-1035-645X>

Research article

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Abstract

Background: This study was conducted to examine the differences in the osteogenic ability of bone marrow mesenchymal stem cells (BMSCs) in patients with hemifacial microsomia on the healthy side versus on the affected side of the mandible.

Methods: Patients with hemifacial microsomia admitted to Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine received bone marrow blood from the healthy and affected sides of the mandible, and BMSCs were cultured to evaluate their osteogenic induction and differentiation activities. Alizarin red (AR) staining was performed to observe the mineralization nodule formation ability of BMSCs. Alkaline phosphatase (ALP) staining was used to measure alkaline phosphatase activity, and the gene expression of the osteogenic-related indicators osteocalcin, osteopontin, Runx-related transcription factor 2, and ALP was detected by real-time fluorescent quantitative PCR. Differences in osteogenic capacity were also explored.

Results: Osteogenesis related staining and Quantitative real-time PCR were performed—the results showed: in AR staining, osteogenesis on the healthy side was significantly better than that on the affected side ($P < 0.001$); in ALP staining, ALP activity of the affected side was significantly lower than that of the healthy side ($P < 0.001$); in the Quantitative real-time PCR, the osteogenesis-related genes ALP, OCN, OPN, and RUNX2 showed high expression on the healthy side and low expression on the affected side ($P < 0.05$).

Conclusions: The osteogenesis ability of BMSCs differed on the healthy and affected mandibular bone in patients with hemifacial microsomia; the osteogenesis ability on the affected side was significantly lower than that on the healthy side. **Keywords:** hemifacial microsomia; bone marrow-derived mesenchymal stem cells; osteogenesis

Background

Hemifacial microsomia (HFM) is defined as a group of malformations caused by developmental defects in the first and second branchial arch, including in the mandible, eye and ear soft tissues, cranial and facial nerves, and even extracranial organs. These facial malformations can cause a variety of phenotypes, such as Goldenhar syndrome, cranial facial microsomia, and oral cavity-mandibular-ear syndrome. HFM is the second most common congenital craniofacial malformation after cleft lip and palate malformation, with incidence rates ranging from 1/26,550 to 1/3500, the average incidence is approximately 1/5600[3], and the male-female ratio is approximately [4].

Since the disease was first reported, researchers in various countries have explored HFM in different fields such as cytogenetics and histoembryology. However, the pathogenesis of HFM is unclear. In terms of histology and embryology, Poswillo suggested that hematoma and abnormalities in neural crest development during the development of the first and second gill arches in embryo led to this malformation, particularly rupture and hemorrhage of the stapes artery[5]. Whereas this pathogenesis has been widely accepted, the specific link between bleeding and deformity remains unclear. In addition, the

administration of retinoids, thalidomide, and other drugs in the early stages of pregnancy, exposure to heavy metals and other substances, administration of vasoactive drugs, multiple pregnancies, diabetes, and vaginal bleeding in the second trimester of pregnancy are associated with the incidence of HFM. Although many studies have been conducted at the genetic level, the disease-causing gene has not been identified.

In this study, BMSCs were cultured from the bone marrow blood from the healthy side and affected side of patients with HFM, and BMSCs were cultured to evaluate their osteogenic induction and differentiation abilities. Differences in the osteogenic ability of bone marrow on the healthy side and affected side of patients with HFM were evaluated by osteogenic staining and gene expression analysis to explore the disease etiology from the origin of osteogenesis.

Materials And Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM), 0.25% trypsin, streptomycin antibiotic, fetal bovine serum (Gibco, Grand Island, NY, USA), osteogenic induction solution (DMEM culture solution containing 10% fetal bovine serum, 10 mmol/L sodium glycerol phosphate, 0.05 mmol/L ascorbic acid, and 10^{-4} mmol/L dexamethasone), Trizol (Invitrogen, Carlsbad, CA, USA), PrimeScript RT Master Mix, SYBR® Premix Ex Taq™ (Takara, Shiga, Japan), alkaline phosphatase staining kit (Biyuntian, Shanghai, China), alizarin red staining kit (Solarbio, Beijing, China), osteogenic primers, and GAPDH reference primers used in this experiment synthesized by Sangon Bioengineering (Shanghai) Co., Ltd. (Shanghai, China) (Table 1) were used in this study

BMSC culture of healthy side and affected side of the mandible of patients with HFM

Patients clinically diagnosed with HFM were selected, and blood samples of the bone marrow from the healthy side and affected side of the patient were collected after obtaining informed consent from the patients or their guardians. Next, 1 mL bone marrow blood was added to 9 mL full medium (DMEM medium containing 10% FBS and 1% cyan-streptomycin antibiotics) and incubated at 37°C with 5% CO₂. The solution was changed twice per week. When the cells reached 80–90% confluence, 0.25% trypsin was used for digestion and passaging of the cells. BMSCs in good cell condition were used in subsequent experiments. Cells from the same passage number were used on both sides. The study protocol was reviewed and approved by the ethics committee of the Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (Approval document number: 2017-382-T279).

BMSC osteogenesis induction

BMSCs from healthy sides and affected side were seeded into 24-well plates (for osteogenic staining, each group was evaluated in triplicate) and 6-well plates (for RNA extraction), cultured in osteogenic

induction medium, and incubated (37°C, 5% CO₂). The medium was changed every three days.

BMSCs osteogenic related staining

ALP staining

BMSCs from the healthy side and affected side were subjected to osteogenic induction for 7 days. The culture medium was discarded, after which the cells were washed twice with PBS, fixed with 4% paraformaldehyde for 20 min, and washed twice with ddH₂O. ALP staining was performed according to the kit instructions. The ALP staining working solution (500 µL) was added to each well, and staining was performed in the dark for 30 min at room temperature. The ALP staining working solution was discarded, the cells were washed twice with ddH₂O to stop the reaction, and the samples were observed under an inverted microscope. ImageJ software (NIH, Bethesda, MD, USA) was used for statistical processing of the images, and data were analyzed with SPSS software 25.0 (SPSS, Inc., Chicago, IL, USA).

AR staining

BMSCs from the healthy side and affected side were subjected to osteogenic induction for 21 days, after which the medium was discarded. The cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 20 min, and washed twice with ddH₂O. Alizarin red stain (500 µL) was added to each well, and the cells were stained for 5–10 min at room temperature. The alizarin red staining solution was discarded, and ddH₂O was used to wash the cells twice to stop the reaction.

Quantitative real-time PCR to detect osteogenic gene expression on healthy and affected sides

BMSCs were subjected to osteogenic induction for 7, 14, and 21 days. The culture medium was discarded, and the cells were washed twice with PBS. Total RNA was extracted on ice using Trizol reagent, cDNA was reverse-transcribed using Takara PrimeScript RT Master Mix, and real-time PCR was performed using SYBR® Premix Ex Taq. For fluorescence quantitative PCR detection, the reaction program was as follows: 95 °C for 10 min; 95 °C for 15 s, 60 °C for 1 min, 40 cycles; 95 °C for 5 s, and 60 °C for 30 s. The relative expression of each osteogenic differentiation-related gene was calculated by the $2^{-\Delta\Delta C_t}$ method, and the experiment was repeated three times.

SPSS25.0 software was used for statistical analysis, and non-paired T test was performed between the healthy and affected groups. There was a statistical difference when $P < 0.05$

Results

Osteogenic staining of BMSCs on healthy and affected sides of patients with HFM

AR staining results

After osteogenesis induction of BMSCs on the healthy side, mineralized nodules were larger and greater in number, whereas mineralized nodules on the affected side were less abundant and sparse. Osteogenesis on the healthy side was significantly better than that on the affected side (Fig. 1a). ImageJ was used to quantitatively evaluate the staining results. At 21 days, AR staining was $53.241 \pm 0.705\%$ in health side group and $23.070 \pm 0.860\%$ in affected side group ($P_{ALP} = 0.000001$), the differences between sides were significant ($P < 0.001$) (Fig. 1b).

ALP staining results

After osteogenesis induction, the color area of BMSCs on the healthy side was large and deep, while the color area of the affected side was small and light. The ALP activity of the affected side was significantly lower than that of the healthy side (Fig. 2a). ImageJ was used to quantitatively evaluate the staining results. At 21 days, AR staining was $42.039 \pm 1.449\%$ in health side group and $17.386 \pm 0.642\%$ in affected side group ($P_{ALP} = 0.00001$), the differences between sides were significant ($P < 0.001$) (Fig. 2b).

Quantitative real-time PCR

BMSCs on the healthy side and affected side of patients with HFM were subjected to osteogenic induction for 7, 14, and 21 days. The osteogenesis-related genes ALP, OCN, OPN, and RUNX2 showed high expression on the healthy side and low expression on the affected side. The differences between sides were significant ($P_{ALP\ 7day} = 0.0004$, $P_{OCN\ 7day} = 0.0352$, $P_{OPN\ 7day} = 0.00002$, $P_{RUNX2\ 7day} = 0.0006$; $P_{ALP\ 14day} = 0.0268$, $P_{OCN\ 14day} = 0.000003$, $P_{OPN\ 14day} = 0.00004$, $P_{RUNX2\ 14day} = 0.00007$; $P_{ALP\ 21day} = 0.0142$, $P_{OCN\ 21day} = 0.0107$, $P_{OPN\ 21day} = 0.0003$, $P_{RUNX2\ 21day} = 0.0006$) (Fig. 3a-c). The results indicated that there were differences in the osteogenesis ability of BMSCs on the affected side of HFM. The osteogenic ability of BMSCs on the affected side was worse than that on the healthy side.

Discussion

HFM is a common congenital malformation of the cranial and maxillofacial regions. Its main manifestation is incomplete mandible development on the side of the infection, which can be accompanied by eyeball dermoid cyst, ear, facial asymmetry, and spinal deformity, and may involve the bone, cardiovascular, urogenital, respiratory, and other systems. Typically, the patient's condition is complicated, and existing treatment methods are mostly symptomatic treatment, creating a social and economic burden for the family and society. HFM shows no obvious familial inheritance. While the disease has been widely studied, the pathogenesis, related pathogenic chromosomal location, and pathogenic genes have not been precisely defined. Thus, there are no effective preventive measures, and thus prenatal diagnosis and possible drug or gene therapy cannot be performed. Therefore, early clarification of the pathogenesis and pathogenic genes of this disease is very important for preventing this disease and developing targeted drugs or gene therapy.

Most of the chromosomal abnormalities detected in different patients with this disease are not consistently identified. Multiple cases of reproducible abnormalities focused on chromosome 5 and 22, respectively, with deletions in the range of 5p13.3-p14[10–14] and 22q11-q13.3[15–18]. In cytogenetics, because of the diverse phenotypes of HFM, it is difficult to clarify the corresponding relationship between specific genes and clinical phenotypes. Additionally, the specific roles of genes in the clinical phenotypes are unclear. Numerous sequencing studies have screened a series of candidate genes such as TCS, SALL1, GSC, MSX, BIRIC, OCLN, SIX1, SIX6, OTX2, SALL4, and EYA1[9]. A genome-wide association analysis (GWAS) of the disease was published in 2016[19]. However, most studies were conducted at the gene screening level, and few follow-up molecular biology or functional tests have been carried out.

In this study, osteogenic induction of BMSCs from the bilateral mandibular bone of patients with HFM was conducted. The osteogenic ability of bilateral BMSCs from patients with HFM differed significantly lower on the affected side than on the healthy side. Thus, the asymmetric development of bilateral mandible in patients with HFM occurs because of differences in osteogenic ability at the level of bone marrow cells. This also suggests that differential gene expression in bilateral BMSCs in patients with HFM is responsible for differences in osteogenic ability, providing a foundation for studies of the genes causing HFM. Previous studies of disease-related genes were carried out by extracting the patient's peripheral blood for gene sequencing and comparing the results to those of the normal population to explore whether mutations in certain genes lead to the disease. Our results showed that BMSCs on the affected side of patients with HFM had a poor osteogenic capacity compared to those on the healthy side, indicating differential expression of genes in the bilateral BMSCs. Gene sequencing should be performed and gene expression levels should be determined to evaluate the pathogenic genes involved in this disease.

Conclusions

In summary, the osteogenic capacity of BMSCs in patients with HFM differed between the affected and unaffected sides, suggesting that there are differences in the gene expression of bilateral BMSCs in our patients. These results provide a foundation for studying the pathogenic genes involved in the disease. Gene sequencing of healthy and affected cells and the detection of gene expression levels may be useful for directly screening pathogenic genes of the disease.

Abbreviations

bone marrow mesenchymal stem cells (BMSCs) hemifacial microsomia (HFM) Alizarin red (AR) Alkaline phosphatase (ALP) osteocalcin (OCN), osteopontin (OPN), Runx-related transcription factor 2 (RUNX2) Dulbecco's Modified Eagle Medium (DMEM)

Declarations

Ethics approval and consent to participate The study was approved by the scientific research ethics committee at Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. Ethical approval number: 2017-382-T279.

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

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Table 1

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

Figures

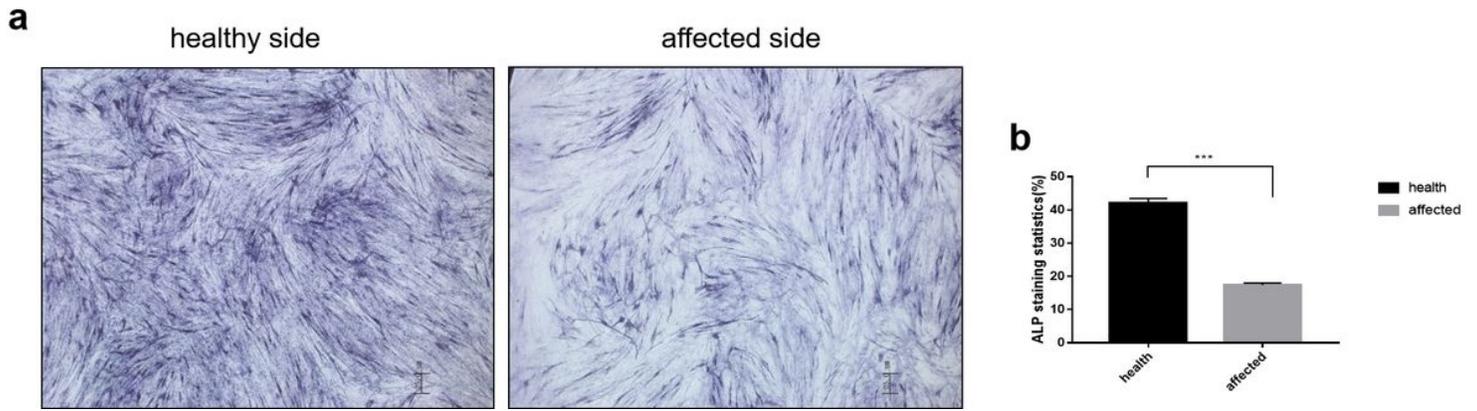


Figure 1

(a)Figure 3 ALP staining images of BMSC osteogenic induction for 7 days on healthy and affected sides of patients with HFM.(b)ALP staining results of BMSC osteogenic induction for 21 days on healthy and affected sides of patients with HFM, *** P < 0.001

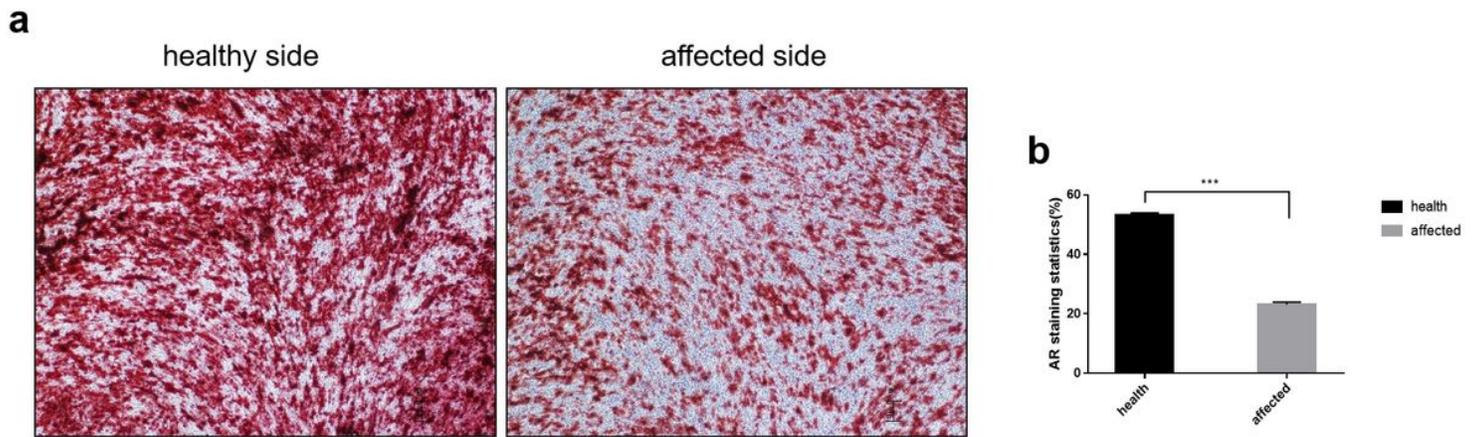


Figure 2

(a)AR staining images of BMSC osteogenic induction for 21 days on the healthy and affected sides of patients with HFM.(b)AR staining results of BMSC osteogenic induction for 21 days on healthy and affected side of patients with HFM, *** P < 0.001

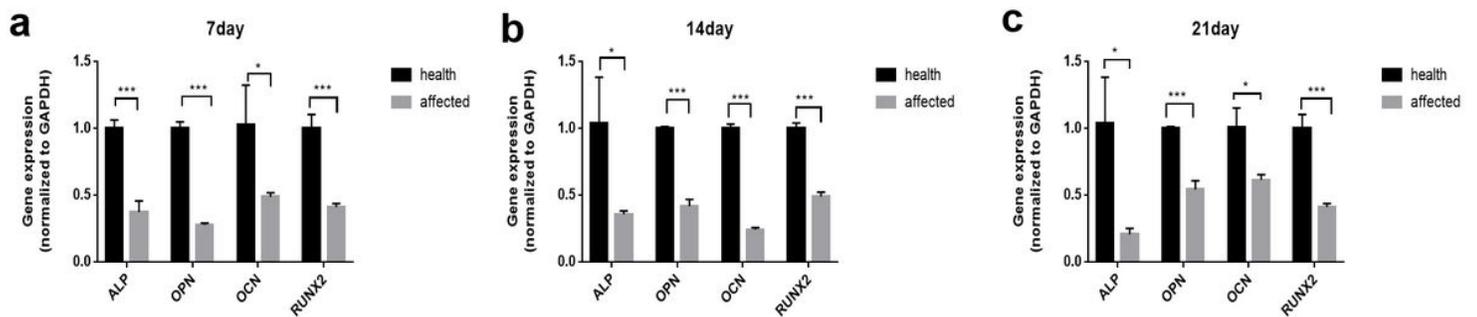


Figure 3

ALP, OPN, OCN, and RUNX2 gene expression levels of osteogenesis-related indicators at 7(a), 14(b), and 21(c) days after osteogenic induction in healthy and affected BMSCs. *P < 0.05, **P < 0.01, ***P < 0.001

Supplementary Files

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- [Table1.xlsx](#)