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An *in vivo* genome wide gene expression study of circulating monocytes suggested GBP1, STAT1 and CXCL10 as novel risk genes for the differentiation of peak bone mass

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ABSTRACT

Peak bone mass (PBM) is an important determinant of osteoporosis. Circulating monocytes serve as early progenitors of osteoclasts and produce important molecules for bone metabolism. To search for genes functionally important for PBM variation, we performed a whole genome gene differential expression study of circulating monocytes in human premenopausal subjects with extremely low ($N=12$) *vs.* high ($N=14$) PBM. We used Affymetrix HG-U133 plus2.0 GeneChip® arrays. We identified 70 differential expression probe sets ($p<0.01$) corresponding to 49 unique genes. After false discovery rate adjustment, three genes [STAT1, signal transducer and activator of transcription 1; GBP1, guanylate binding protein 1; CXCL10, Chemokine (C-X-C motif) ligand 10] expressed significantly differentially ($p<0.05$). The RT-PCR results independently confirmed the significantly differential expression of GBP1 gene, and the differential expression trend of STAT1. Functional analyses suggested that the three genes are associated with the osteoclastogenic processes of proliferation, migration, differentiation, migration, chemotaxis, adhesion. Therefore, we may tentatively hypothesize that the three genes may potentially contribute to differential osteoclastogenesis, which may in the end lead to differential PBM. Our results indicate that the GBP1, STAT1 and CXCL10 may be novel risk genes for the differentiation of PBM at the monocyte stage.

Introduction

Osteoporosis is a serious health problem of excessive skeletal fragility leading to low trauma fractures among the elderly [1]. Low bone mass in the elderly is determined by peak bone mass (PBM) in young adults and subsequent bone loss with aging later in life [2]. Attainment and maintenance of high PBM in young and middle aged adults is of primary importance in protecting against late-life osteoporosis.

Peripheral blood monocytes can serve as early precursors of osteoclasts [3–6]. *In vitro*, multinuclear mature functional osteoclasts can be derived from circulating monocytes when placed in a suitable microenvironment [3,6]. Blood monocytes also produce a wide variety of factors involved in bone metabolism, such as interleukin-1, tumor necrosis factor-α (TNF-α), interleukin-6, platelet-derived growth factor, transforming growth factor-β, and $1,25(OH)_2D_3$ [7]. All osteoclasts in peripheral skeleton [4,8] and a considerable amount of osteoclasts in the central skeleton [9] come from circulating monocytes. Recently, Liu et al. [10] studied peripheral blood monocytes for differential expression genes (DEGs) between elderly women with extremely low *vs.* high bone mineral density (BMD). They suggested a novel pathophysiological mechanism for osteoporosis that is characterized by increased recruitment of circulating monocyte into bone, and enhanced monocyte differentiation into osteoclasts.

The course of differentiation from circulating monocytes to osteoclasts contains a series of osteoclastogenic processes including monocytes' motility, adhesion, transendothelial migration, prodifferentiation, proliferation, chemotaxis, adhesion, activation, and maturation etc. Therefore, we hypothesize that the DEGs at the precursors of osteoclasts functionally involved in the osteoclastogenic processes may contribute to differential osteoclastogenesis, which may in the end lead to differential PBM.

Microarray technology is a high-throughput and powerful tool in identifying and comparing the patterns of gene expression. The technology has substantially improved in recent years and shown reliable and repeatable results across various labs and platforms [11,12].

Here, we analyzed the gene expression profile of circulating monocytes (via the Affymetrix HG-U133 plus2.0 GeneChip® array) in human subjects with extremely low *vs.* high PBM to identify DEGs functionally potentially relevant to the differentiation of osteoclastogenesis.

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Materials and methods

Subjects

The study was approved by Hunan Normal University, ChangSha, China. All the recruited volunteers signed informed consent form before entering this project. All the study subjects belong to Chinese Han ethnic group. We first recruited 878 healthy Chinese premenopausal females aged 20-45 y with an average of 27.3 y when PBM is attainted and maintained [13,14]. Then, we distributed the total sample according to the hip *Z*-score of PBM. From the bottom 100 and top 100 subjects of the PBM phenotypic distribution, we selected 12 subjects (Mean *Z*-score ± SD = -1.72 ± 0.60) and 14 (Mean *Z*-score ± SD = 1.57 ± 0.57) with extremely low and high PBM for further DNA microarray experiments. We adopted strict exclusion criteria detailed by Liu et al. [10], to minimize any known potential confounding effects on the variation of bone phenotype. Briefly, patients with chronic diseases/conditions that may potentially affect bone mass were excluded. These diseases/conditions included chronic disorders involving vital organs (heart, lung, liver, kidney, brain), serious metabolic diseases (diabetes, hypo- or hyperparathyroidism, hyperthyroidism), other skeletal diseases (Paget's disease, osteogenesis imperfecta, rheumatoid arthritis), chronic use of drugs affecting bone metabolism (corticosteroid therapy, anticonvulsant drugs), and malnutrition conditions (chronic diarrhea, chronic ulcerative colitis).

BMD measurement

BMD (g/cm^2) for the lumbar spine (L1-4) and total hip (femoral neck, trochanter, and intertrochanter region) was measured with a Hologic 4500W dual energy X-ray absorptiometer (DEXA) scanner (Hologic Corporation, Waltham, Massachusetts, USA). The machine was calibrated daily. The coefficient of variation of BMD values from the DEXA measurements, obtained from 7 individuals repeatedly measured five times, of the DEXA measurements was 0.80% at the hip.

Experiment procedures

Monocyte isolation

A monocyte negative isolation kit (Dynal Biotech Inc., Lake Success, NY) was used to isolate circulating monocytes from 50 ml whole blood following the procedures recommended by the manufacturer. The kit can deplete T cells, B cells, and natural killer cells from mononuclear cells, leaving only monocytes untouched and native and free of the surface-bound antibody and beads. This is of particular importance as binding antibody-coagulated beads to the cell surface may activate the cells and thus change their gene expression profiles. The purity of the isolated monocyte sample was examined by BD-FACScalibur flow cytometry (BD Biosciences, San Jose, CA USA) with fluorescence labeled antibodies PE-CD14 and FITC-CD45. The purity of the isolated monocyte ranged from 70%-90% in our samples.

Total RNA extraction and microarray assays

Total RNA from monocytes was extracted using Qiagen kit (Qiagen, Inc., Valencia, CA) following the procedures recommended by the manufacturer. Experimental procedures for microarray assays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Briefly, RNA was converted to double-stranded cDNA. *In vitro* transcription was performed to produce biotin-labeled cRNA (BioArray HighYield RNA Transcription Labeling Kit; Enzo Diagnostics). Biotinylated cRNA was cleaned, fragmented, and hybridized (Affymetrix Genechip Hybridization Oven 640) to Affymetrix HG-U133 plus2.0 GeneChips, containing about 38,500 human genes as represented by analysis of over 47,000 transcripts and variants. Then, microarrays were washed (Affymetrix Fluidics Station 450), stained with phycoerythrin-streptavidin, and scanned using an Affymetrix scanner (Gene array Scanner 3000).

Table 1
Basic characteristics of the studied subjects

Trait	All	Top 100 subjects	Bottom 100 subjects	Low PBM group for microarrays	High PBM group for microarrays	*p* value
Number	878	100	100	12	14	
Weight (kg)	50.7 ± 6.2	47.7 ± 5.5	54.4 ± 6.6	51.5 ± 7.3	55.8 ± 5.7	0.10
Height (cm)	158.2 ± 5.1	156.5 ± 4.9	159.7 ± 5.2	158.9 ± 4.4	158.9 ± 5.3	0.98
Age (years)	27.3 ± 4.8	27.1 ± 4.6	27.5 ± 5.0	25.3 ± 3.1	28.7 ± 4.7	0.045
PBM (g/cm^2)						
Hip	0.87 ± 0.10	0.71 ± 0.04	1.05 ± 0.05	0.70 ± 0.06	1.03 ± 0.05	0.0001
Spine	0.94 ± 0.10	0.85 ± 0.08	1.06 ± 0.09	0.85 ± 0.07	1.04 ± 0.09	0.0001

Notes:
1. Values are presented as means ± standard deviation.
2. Top 100 and bottom 100 subjects means the top 100 and bottom 100 subjects of the PBM phenotypic distribution (by hip *Z*-score).
3. *p* value is the statistic level of *t*-test in the low *vs.* high hip PBM groups for microarray experiments.

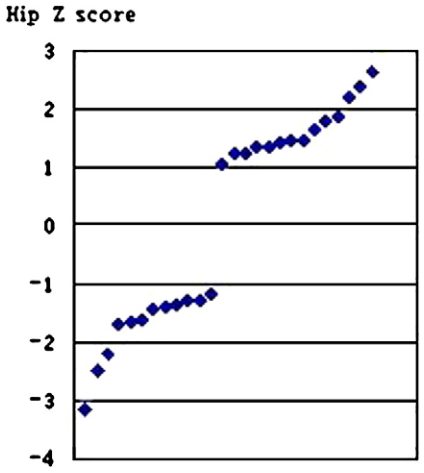

Fig. 1. The phenotypic distribution of 14 and 12 subjects with extremely high *vs.* low PBM.

Real-time RT-PCR

We used two-step real-time RT-PCR to confirm the selected DEGs, i.e., reverse transcription for synthesis of cDNA from total RNA followed by real-time quantitative PCR. RT reactions were performed in a 30 μL reaction volume, containing 3 μL 10× PCR Buffer II, 6.6 μL 25 mM $MgCl_2$, 6 μL dNTPs, 0.75 μL MULV reverse transcriptase, 0.6 μL RNase inhibitor, 1.5 μL Oligo d(T), 0.6 μg total RNA and water to 30 μL. All the above reagents were supplied by Applied Biosystems (Foster City, CA). Reaction conditions were as follows, 10 min at 25 °C, 30 min at 48 °C, 5 min at 95 °C.

Real-time quantitative PCR was performed in 20 μL reaction volume using standard protocols on an Applied Biosystem's 7000 Sequence Detection System. Briefly, 2 μL cDNA was mixed with 2 μL 10× TaqMan Buffer A, 4.4 μL 25 mM MgCl2, 0.4 μL 10 mM dATP, 0.4 μL 10 mM dCTP, 0.4 μL 10 mM dGTP, 0.4 μL 20 mM dUTP, 0.2 μL AmpErase UNG, 1 μL Assays-on-DemandTM Gene Expression Assay Mix (contains forward and reverse primers and labeled probe), 0.4 μL GAPDH Probe, 0.4 μL GAPDH Forward Primer, 0.4 μL GAPDH Reverse Primer, 0.1 μL AmpliTaq Gold DNA Polymerase (5.0 U/μL) and water to 20 μL. The thermocycling conditions are as follows: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C plus 1 min at 60 °C. The thermal denaturation protocol was run at the end of the PCR to determine the copy number of products that were presented in the reaction. All reactions were run in triplicates and included "no template" controls for each gene. As the TaqMan Gene Expression Assays all have amplification efficiencies very close to one, we did not perform validation experiments to test the equality of the amplification efficiencies between the target genes and the reference GAPDH gene.

Data analyses

GCOS 1.2 (GeneChip Operating Software) was used to process the probe-level raw data. We used the RMA (Robust Multiarray Average) algorithm [15] molded in R package to transform the probe-level raw data into gene expression data. RMA can give most reproducible results and show the highest correlation coefficients with RT-PCR data among currently available algorithms [16]. Based on the expression data generated with the RMA algorithm, Matlab software package was used to perform student *t*-test to compare the expression signals in subject groups with extremely low *vs.* high PBM to identify DEGs. To account for multiple testing, a false discovery rate (FDR) method, Benjamini and Hochberg stepwise procedure [17], was used to generate adjusted p values. To better understand the differential expression profile of circulating monocyte between subjects with extremely low *vs.* high PBM, gene ontological analyses of the DEGs were performed by Onto-Express [18], available at http://vortex.cs.wayne.edu/ontoexpress/. Pathway-Express [19] was performed to identify potentially interesting pathways for these DEGs.

Real-time RT-PCR data were generated with the ABI Prism 7900 sequence detection system software (Applied Biosystems). The cycle number at which the reaction crossed a predetermined cycle threshold (CT) was identified for each gene, and the expression of each target gene relative to GAPDH gene was determined using the equation 2-ΔCT, where ΔCT = (CTTarget Gene-CTGAPDH). Based on the relative gene expression, we performed student's *t*-test to validate the DEGs between the discordant PBM groups.

Results

Table 1 lists the basic characteristics of the studied sample. We can find significant difference of hip PBM between the low and high hip PBM groups for microarray experiments. Fig. 1 intuitively shows that