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Differential Gene Expression Pattern in Osteoclast Precursor Cells of Indian Postmenopausal Women with and Without Osteoporosis: A Microarray Based Study

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Abstract

Background: Osteoporosis is a multifactorial disease with strong genetic and epigenetic component. In spite of enormous candidate gene association studies, the etiology and molecular mechanism of disease is not fully known. For identification of new markers of osteoporosis which could be vital in diagnosis and prognosis of disease, genome-wide microarray expression approach was employed.

Methods: Osteoclast precursor cells were sorted from circulating monocytes of osteoporotic and non-osteoporotic post menopausal females with similar life-style and year after menopause. Following microarray experimentation, gene enrichment analysis was performed on significant DEGs using Database for Annotation, Visualization and Integrated Discovery (DAVID) tool. Top 10 novel genes were further used for construction of protein-protein interaction network using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database. To validate microarray gene expression pattern, Real time-PCR was performed.

Results: A total of 269 genes were found to be differentially expressed between disease and normal groups, of which 138 were observed to be up regulated and 131 down regulated. Furthermore, novel LHX1 gene was observed to be up regulated and its interaction with BMP4 protein was observed. Three known genes for osteoclastogenesis (viz., CX3CL1, ACP5 and CSF1) were found to be up- regulated. Similar pattern of gene expression have been obtained using RT-PCR.

Conclusion: Significant enrichment of PI3K-Akt and TGF-ß signaling pathways involving DEGs was found in postmenopausal Asian Indian women. Moreover, up regulated LHX1 gene was discovered as novel gene which may have a role in pathogenesis of osteoporosis and can be used for diagnosis and prognosis along with known osteoporotic markers.

Keywords: Osteoporosis; Monocytes; Osteoclast precursors; Microarray; Differential gene expression; LHX1 gene

Introduction

Osteoclasts play a pivotal role in the bone resorption. Generally, the formation and resorption of the bone is tightly regulated by nutrition and intrinsic factors. However, when the resorption of the bone prevails over its formation, it leads to a skeletal disorder, osteoporosis. Osteoclast precursors are hematopoietic in origin and differentiate into osteoclasts to resorb bone [1]. Osteoclasts are derived from macrophage colony-stimulating factor (M-CSF)-dependent blood precursors, including monocytes present in the peripheral blood mononuclear cell (PBMC) population. Osteoclast precursors circulate in the blood stream to find the correct site (bone surface) for osteoclastogenesis [2]. If the bone surface is not available, differentiation of these precursors into osteoclasts may become insensitive and regain osteoclastogenesis when bound to bone. So adhesion of osteoclast precursors to bone dictates their fate [3]. However, the most likely candidate for the osteoclast precursor in PBMC population is the CD14 marker, which is strongly expressed on monocytes. CD14+ cells are putative osteoclast precursors in the peripheral blood [4,5]. Like osteoclasts, immune cells also originate from hematopoietic stem cells and a link between immune cells and bone cells has revealed that along with RANK/RANKL/OPG system, activated T cells may both positively and negatively regulate bone resorption [6]. Apart from these, hormones, interleukins, growth factors, nutrition, physical activity and lifestyle play a major role in the development, differentiation and maturation of osteoblasts and osteoclast precursor cells, suggesting that bone resorption is being influenced by both local and systemic factors [7]. Thus, osteoporosis is a multifactorial disease in which genetic and epigenetic factors along with lifestyle play an important role [8].

For primary osteoporosis along with these factors, menopause status is the most vulnerable factor in women. Deficiency of estrogen with simultaneous increase in the FSH level accelerates bone loss inducing osteoporosis [9]. Estrogen depletion recruits large number of osteoclast precursors to activate osteoclastogenesis [10,11]. Reduced

enstrogen inhibits apopotosis of osteroclasts and facilitates the formation of osteoclasts by inducing osteoclast precursor sensitivity to receptor activator of nuclear factor kappa-B ligand (RANKL) induced osteoclastogenesis. Osteoclast precursors up regulate c-Jun activating kinase (JNK) resulting into increased bone resorption [12,13].

Although association studies have shown the involvement of genetic factors along with several candidate genes either singly or in group, the etiology of osteoporosis is not clearly understood.

To address this complex question researchers have also screened differential gene expressions of bone cells using high throughput microarray [14,15]. In most of these studies either the effect of estrogens, corticosteroids oxidative stress or some drugs on osteoblasts have been analyzed [16,17]. Only a handful of studies have focused on the gene expression pattern of osteoblasts obtained from bone marrow or bone biopsies or cell lines [16-18]. Further, some studies have also examined the gene expression profile of premenopausal women and primary osteoporosis [17,19]. Liu et al. (2005) studied the genome wide differential gene expression on circulating monocytes from osteoporotic and non-osteoporotic postmenopausal Caucasian females. However, they did not take into account the years lapsed after menopause, life style, diet and medication of the participating women, which influences the pattern of gene expression to a great extent [20]. Moreover, their study included Caucasian and Asian Chinese populations. We know that factors such as duration of exposure to sun, diet, lifestyle etc. of Indians is completely different from Caucasian or Chinese populations. Very few reports are available on the status of osteoporosis in Indian women. Moreover, these studies are based on survey and questionnaire about lifestyle, sun exposure, nutrient intake etc. and their impact on Bone Mineral Density (BMD). These studies have shown that, vitamin D deficiency is a major factor for reduced BMD and high prevalence of osteoporosis in Indian population [21,22]. In Indian women from low income group with low nutrient diet, body weight, age and menopause are important determinants of BMD [23].

As per our knowledge, there are no studies on differential gene expression of osteoclast precursors and identification of osteoporosis specific genes in Asian Indian women. Hence, in the present study, we first tried to identify Differentially Expressed Genes (DEGs) in osteoclast precursor cells derived from Indian postmenopausal women having high bone mineral density (as a control) and primary osteoporosis. Further, we validated microarray data of five potential genes using reverse transcription polymerase chain reaction (RT-PCR). This is the first genome-wide expression study on ex situ human osteoclast precursor cells relating to etiology of osteoporosis in Indian postmenopausal women who have had almost similar life style and years lapsed after onset of menopause.

Material & Methods

The study was approved by the ethics committee of Savitribai Phule Pune University, Pune. Biological samples were collected after obtaining a written consent from female volunteers.

Bone mineral density (BMD) measurement

Bone mineral density (g/cm^2) was measured at femoral neck and lumbar spine (L2-L4) using a dual X-ray absorptiometry (DEXA; 24). The osteoporotic and non-osteoporotic conditions were decided using world health organization (WHO) guidelines stating T score above -1 SD is non-osteoporotic and below -2.5 SD is osteoporotic condition. Based on this guideline for T score, participating subjects (n=72) were grouped into non-osteoporotic (n=36) and osteoporotic (n=36) to investigate DEGs pattern between them.

Subjects

All participants were genetically unrelated postmenopausal females from Pune (18.5204°N, 73.8567°E), India. Subjects with secondary osteoporosis and chronic skeletal diseases (rheumatoid arthritis, osteogenesis etc.), metabolic disorders (diabetes and thyroidism), chronic conditions of internal organs (liver, kidneys etc.) and endocrine disorders undergoing hormonal therapies (hormone replacement, bisphosphonate, corticosteroid therapy) were excluded as these conditions possibly affect bone metabolism. Individuals fitting to inclusion criteria were recruited and information regarding family history of bone related diseases, medical history of hysterectomy, ovariectomy, menstrual history, dietary habits, physical activity, lifestyle (alcohol, smoking etc.) and calcium supplementation, number of children and duration of lactation was collected. Median body mass index (BMI; kg/m^2) and years after menopause matched females (n=8) with osteoporosis (n=4) and non-osteoporosis (n=4) were selected for microarray analysis. All the subjects, (normal=32 and osteoporosis=32) in the age group of 45-60 years were selected for the gene expression validation study by RT-PCR.

Experimental Procedure

Osteoclast precursor cell sorting

Whole blood (25 ml) from each selected subject was collected in EDTA vials. This was divided into two vials: one with 15ml for CD14+ cell sorting and second with 10 ml for total RNA isolation. PBMCs of were isolated from whole blood of both vials by fractionating on Hypaque 1077. Isolated PBMCs from 15 ml blood vial were resuspended in 1 ml of isolation buffer (Ca^{2+} and Mg^{2+} free PBS supplemented with 0.1% BSA and 2 mM EDTA, pH 7.4) and cell count was taken. An average of ~ 1.5×10^7 cells/ml were obtained and subjected to $CD14^+$ sorting as CD14 is a putative osteoclast precursor in the peripheral blood [4,5]. CD14+ cells were sorted from circulating monocytes using Dynabeads® CD14 positive isolation kit (INVITROGEN, MA, USA) following the manufacturer's protocol [25]. As these Dynabeads are coated with primary monoclonal antibody exclusive for CD14 membrane antigen, it efficiently eliminates T cells, B cells, natural killer cells, $CD16^+$ and granulocytes and gives ~95% pure $CD14^+$ cells.

Total RNA was isolated from these $CD14^+$ cells using Trizol reagent (LIFE TECHNOLOGIES, CA, USA) and was purified using RNeasy mini kit (QUIGENE, Hilden, Germany). Total cDNA was synthesized from 1µg total RNA using Verso cDNA synthesis kit (THERMO FISHER SCIENTIFIC, MA, USA). Reverse transcription reaction was set according to manufacturer's protocol followed by RT-PCR for c-FMS and RANK genes which are markers for osteoclast precursors. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. Following thermal cycler program was used: 95°C for 10 min, 95°C for 30 s, 58°C for 1 min, 60°C for 35 s, 95°C for 15 s, 60°C for 1min and 95°C for 15 s with 40 cycles. SYBR green chemistry was used to assess the melt curves on Step one plus (APPLIED BIOSYSTEMS, CA, USA) thermal cycler. The composition of PCR was 50 ng cDNA in a total reaction volume of 10µl using 5 µl DyNAmo ColorFlash SYBR Green qPCR Kit (THERMO FISHER SCIENTIFIC, MA, USA) and 10 pM each of forward and reverse

primers. Following primers were used for genes used as markers for osteoclast precursors- GAPDH- F: TGGGGAAGGTGAAGGTCGGA, R: GGGATCTCGCTGCTGGAAGA, c-FMS- F: CCTAGCACAGGAGGTGGTCGT, R: GGTGTTGTTGTGTTGGAGGA, RANK- F: CCTGAAGAAGAAGCCAGCAG, R: CGTAGACCACGATGATGTCG.

GeneChip hybridization

After confirming presence of osteoclast precursor specific markers, total RNA from control (n=4) and osteoporotic (n=4) individuals was used for in-vitro transcription. Biotin labeling was carried out using CY3 dye of Agilent quick-Amp labeling kit and amplified using T7 promoter. The Quality of Biotinylated cRNA was assessed by Nanodrop and all the samples were found to be suitable for further hybridization. Integrity of RNA was checked by assessing the ratio of 18S and 28S ribosomal RNA intensity peaks (Agilent 2100 Expert Bioanalyzer; Table 1). Human Genechip® Agilent's in-situ hybridization kit 5190-6420 was used which covers approximately 60K oligonucleotide probes. Experimental procedure for microarray hybridization and scanning was performed according to manufacturer's protocol (AGILENT TECHNOLOGIES, CA, USA).

Sample no. of participants	'T' score [SD]	RNA concentration [ng/µl]	Absorbance value [260/280]	RNA Integrity Number
Normal [n = 4]				
1	-0.1	551.2	1.9	7.2
2	-0.5	780.7	1.78	9.7
3	-0.9	547.6	1.68	6.9
4	1.2	199.6	1.85	7.2
Osteoporosis [n = 4]				
5	-2.7	298.8	1.89	7.1
6	-2.7	454.9	2.05	5.5
7	-3.3	473.3	2.04	7.2
8	-3.5	386.5	1.94	9.1

Table 1: Anthropometric and isolated RNA details of eight female subjects (age group 50 - 60 yrs) used for microarray study. Note: Sample no. of participant indicates 1-4 are normal or non osteoporotic individuals and 5-8 are osteoporotic individuals.

Statistical and software analysis

The data generated by microarray hybridization was intra-array normalized using Genespring GX (version: 13.0) software. For inter-array normalization, median values of 4 normal and 4 osteoporosis females across all genes (spots / entity) were considered. From the median value of each entity of osteoporosis, the median value of normal was subtracted. This normalized data was used for log two-fold change (FC) calculation. The relative gene expression achieved through RT-PCR was computed using following formula:

Relative gene expression = $2^{-\Delta Ct}$,

where ΔCt= [Ct (target gene)-Ct (GAPDH)]

Difference in the expression pattern of five genes between control and osteroporosis subjects was analyzed using One- way analysis of variance (ANOVA). Pearson's chi-square test was carried out to assess the significant difference in expression of genes using RT-PCR among normal and disease groups. Microarray data was analysed using GeneSpring GX version: 13.0 software (AGILENT TECHNOLOGIES). Raw data files from Agilent- AMADID: 39494 platform were subjected to RAM (Robust Multichip Averaging) algorithm to normalize the data. The quantile normalization has been implemented with median of all samples taken for baseline transformation. Gene expression profiles of osteoporotic as well as control subjects were studied by generating a differentially expressed genes (DEGs) list. A paired t test was applied using asymptotic method on the normalized data assuming equal variances, incorporating Benjamini and Hochberg False Discovery Rate (FDR) multiple-testing correction set at a rate of 0.05

Screening of differential genes

Differentially expressed genes between osteoporotic and normal samples were identified using Genespring GX version: 13.0 software. False discovery rate of 0.05 was used as a cut off criterion. Differentially expressed genes of protein probes were also filtered out. This normalized and processed microarray data for DEGs of osteoclast precursors was then submitted to NCBI- Gene Expression Omnibus (GEO) repository. The NCBI-GEO accession number is: GSE100609.

Gene cluster analysis

Initial gene clustering of osteoporosis and control groups was carried out using ArrayMining online tool (http://arraymining.net/). The gene expression data matrix was analyzed for hierarchical clustering which builds cluster of genes with similar pattern of expression.

GO enrichment analysis

Gene ontology is a common approach for functional studies of large scale genomic data. Gene enrichment analysis of DEGs was performed using web-based program - Database for Annotation, Visualization and Integrated Discovery (DAVID version: 6.8). After uploading the official symbols of DEGs onto the DAVID website (https://david.ncifcrf.gov/), the online functional annotation tool was applied to identify the pathways related to these genes for 'Homo sapiens' in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Enrichment of pathways for DEGs on a KEGG pathway was considered statistically significant only if the Bonferroni-adjusted $p<0.05$. DAVID endows with a complete set of functional annotation tools to search high-throughput and integrated data-mining environment for the osteoporosis related and predicted genes.

Protein-protein interaction (PPI) network analysis

Protein-Protein interaction network of top ten DEGs was constructed using search tool for the retrieval of interacting genes/proteins (STRING) database version: 10.0 (http://string-db.org/). It is an online biological database and web resource which provides known direct (physical) and indirect (functional) protein-protein interactions.

Transcription factor analysis

Prediction of transcription factors regulating these DEGs were analyzed using TFBS tool of DAVID program. Additionally, prediction of target genes in a biological system and their regulation by known transcription factors was analyzed using TFactS online tool (www.tfacts.org).

Real time RT-PCR analysis

Isolated PBMCs from 10 ml blood were used for total RNA isolation using Trizol following cDNA synthesis from 1µg total RNA using Verso cDNA synthesis kit (Thermo scientific). Apart from eight samples used for microarray hybridization reaction, others (normal = 32 and osteoporotic = 32) were used for quantitative real time PCR. Five DEGs viz, Colony Stimulating Factor 1(CSF1), Acid Phosphatase 5 (ACP5), Chemokine (C-X3-C motif) Ligand 1(CX3CL1) and LIM Homeobox 1 (LHX1) based on their role in the bone metabolism and statistical significance were selected and their microarray gene expression was validated by RT-PCR using GAPDH as an endogenous control. Quantitative RT PCR was performed on Applied Biosystems Step one plus machine in total of 10ul of reaction volume with 50ng cDNA using 5µl DyNAmo ColorFlash SYBR Green qPCR Kit (Thermo scientific) and 10pM each of forward and reverse primers. The primers were GAPDH- F: GGAGTCAACGGATTTGGT, R: GTGATGGGATTTCCATTG, CSF1- F: AAGTTTGCCTGGGTCCTCTC, R: CATACGGGCAGCTGGGTC, ACP5- F: CTTTCTACCGCCTGCACTTC, R: GTTTCTTGAGCCAGGACAGC, CX3CL1- F: CTCCTCTTCTGCCTGGGGGTG, R: AGGAGTTCACACGGGCACCA and LHX1- F: GGGGGCAACTACGACTTCTT, R: GATGACGGCACGAAGGGTAG. The conditions for PCR were – 95°C for 10 min, 95°C for 30 s, 58°C for 1 min, 60°C for 35 s, 95°C for 15 s, 60°C for 1 min and 95°C for 15 s with 40 cycles. All the reactions were run in triplicate along with negative control for each gene.

Results

DEGs suggested by microarray analysis

Microarray data were processed and normalized to obtain DEGs among osteoporotic subjects. A total of 556 differentially expressed genes with threshold of $p<0.05$ were filtered. Following exclusion of protein probes, 269 DEGs were screened of which 138 were up regulated and 131 down regulated genes. The hierarchical clusters based on 15 DEGs ensured that the screened genes have significant differences between osteoporotic and normal subjects. Further, principal component analysis of the samples also gave similar results for two separate clusters (Figure 1).

Prediction of novel osteoporosis genes

Osteropotic genes were chosen based on two criteria i) potential functional relevance of DEGs to bone metabolism and ii) statistical significance. With the first criterion, four genes namely, CX3CL1, CSF1, ACP5 and SMAD2 were screened out for their significant involvement in osteogenesis. CSF1, ACP5 and CX3CL1 are known osteoclastogenesis genes and were up-regulated. Though SMAD2 was significantly down-regulated, further emphasize on SMAD 2 was not given as it's an osteoblastogenesis specific marker. Based on the second crieterion top ten up-regulated and down-regulated genes were screened out as novel. First twenty DEGs with their corresponding fold change and p-value obtained by microarray are discussed in the supplementary Table 1.

Interaction of these genes in the bone metabolism was evaluated using STRING database, which indicated the association of LHX1 with bone metabolism. LHX1 gene was also involved in osteoblast development (GO:0002076), differentiation (GO:0001649) and its regulation (GO:0045667) with false discovery rates below 0.05. LHX1 protein also showed its interaction with BMP4, CSF1, ACP5 and SMAD2 which are known potential osteogenesis markers.

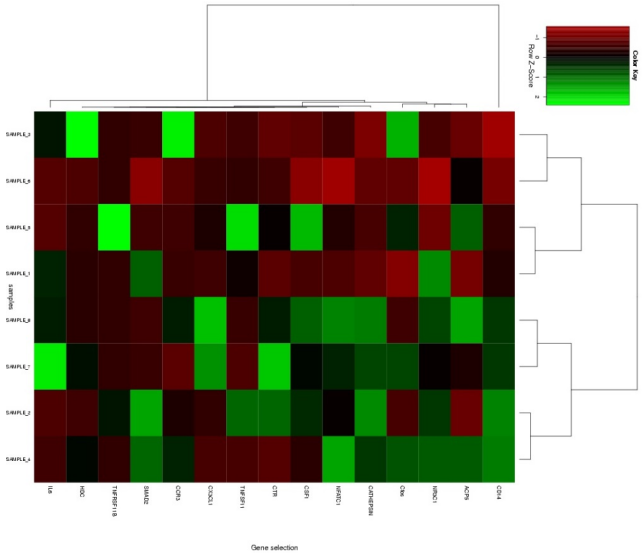

Figure 1: 2D Clustering of osteoporotic and normal samples based on 15 differentially expressed genes characteristic of osteoclast precursors and involved in early osteoclast differentiation using ArrayMining - online microarray data mining program. Horizontal dendogram depicts osteoporosis and non-osteoporosis subjects forming two separate clusters represented in columns. Vertical dendogram shows selected genes clustering together based on their expression value.

Protein protein Interaction analysis

Analysis of protein-protein interactions (PPIs) was performed on DEGs to construct network of their interaction using STRING software. The prediction of critical genes for osteoporosis was done and combined interactions were expressed as histogram. The hub nodes of interaction for osteoporosis (namely SMAD2, BMP4, RLIM and LHX1) were identified (Figure 2). Node LHX1 ($p=0.0002$) was observed to be interacting with maximum number of genes involved in disease (SMAD2, BMP4, FOXA2, SFRP5, LDB2, LDB1, SHH and RLIM) with PPI enrichment score 0.000312 implicating LHX1 as the key gene associated with osteoporosis among Asian Indians. Thus, LHX1 was considered as fourth gene along with CSF1, CX3CL1 and ACP5 as probable markers in the predisposition of postmenopausal Asian Indian women for osteoporosis and for validation study by RT-PCR.

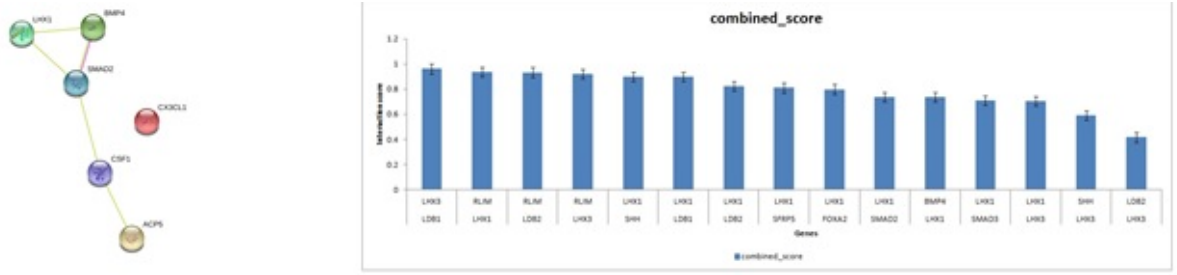

Figure 2: (a) Protein-protein interaction of selected four genes obtained using STRING database (b) combined score of interaction between LHX1 and other proteins.

Since the expressions of majority of 269 DEGs (listed in supplementary Table 1) have not been validated with real time PCR, significance (i.e. the p-value) of their differential expression is interpreted with caution.

Functional analysis of DEGs in osteoporosis

Gene ontology analysis was performed to know biological processes associated with DEGs using DAVID. In the functional cluster analysis, eight clusters were formed of which three clusters with enrichment score >0.05 were selected. We found that DEGs were mostly involved in the TGF-beta receptor signalling pathway (enrichment score 1.04), SMAD protein signal transduction (enrichment score 0.71), cell adhesion (enrichment score 0.58), etc. The genes which were significantly involved in these processes were SMAD2, SKI (TGF-beta receptor signalling pathway), INHBA (SMAD protein signal transduction) EMP2, CX3CL1, NCAN (Cell adhesion), etc.

Annotation [pathway/process]	Overlap size	list of genes
hsa01100:Metabolic pathways	14	AUH, SEPHS2, PYCRL, POLR1E, POLR1A, LIPT1, HYAL1, GALT, CES1, ASS1, UGCG, UGDH, NAT1, CDS2,
hsa04080:Neuroactive ligand-receptor interaction	10	F2RL1, ADRB1, ADRB3, CHRM4, GRIN2C, GRIN3B, PTAFR, PRLR, SSTR4, S1PR4,
hsa04060:Cytokine-cytokine receptor interaction	8	CX3CL1, ACVR1, CSF1, IL17A, KDR, PRLR
hsa04151 PI3K-Akt signalling pathway	8	COMP,CSF1,FGF11,FGF8,ITGB8,KDR,PRLR,TNC
hsa05200:Pathways in cancer	7	RAD51, SMAD2, FGF11, FGF8, MMP1, RET
hsa04144:Endocytosis	7	SMAD2, ADRB1, ADRB3, CHMP1B, KDR, PARD6A, RET, SMAD2
hsa04015 Rap1 signalling pathway	6	CSF1, FGF11, FGF8,KDR, PARD6A, SIPA1
hsa04810:Regulation of actin cytoskeleton	5	BAIAP2, CHRM4, FGF11, FGF8, ITGB8
hsa04350:TGF-beta signalling pathway	4	SMAD2, SMAD5, ACVR1, INHBA,
hsa04014 Ras signalling pathway	4	CSF1, FGF11, FGF8, KDR
hsa04510 Focal adhesion	4	COMP, ITGB8, KDR, TNC
hsa04550 Signalling pathways regulating pluripotency of stem cells	4	ACVR1,INHBA, SMAD2, SMAD5
hsa05323 Rheumatoid arthritis	4	ACP5, CSF1, IL17A, MMP1

Table 2: list of genes involved in the GO enrichment pathway analysis using KEGG database.

Pathway analysis

The pathways for osteoporosis were analyzed using KEGG Mapper v2.8, a web based database. Our DEGs were associated with pathways like PI3k-Akt signaling pathway (CX3CL1, ACVR1, CSF1, IL17A, etc.), RAP1 & RAS signaling pathway (CSF1, FGF11, FGF8), TGF-beta signaling pathway (SMAD2, SMAD5, ACVR1, INHBA), which are known to participate in osteogenesis. A list of DEGs involved in the pathways is summarized in Table 2.

Validation of selected DEGs with RT-PCR

Differential expression of CX3CL1, CSF1, ACP5 and LHX1 was validated using qRT PCR. For this purpose, RNA isolated from CD14+ cells as well as from PBMCs was used. The pattern of relative gene

expression was (complementary) with the microarray data indicating significant up regulation of LHX1 gene. A significant increase in the CSF1 gene expression could be because of fewer years (4 years) after menopause onset (Figure 3).

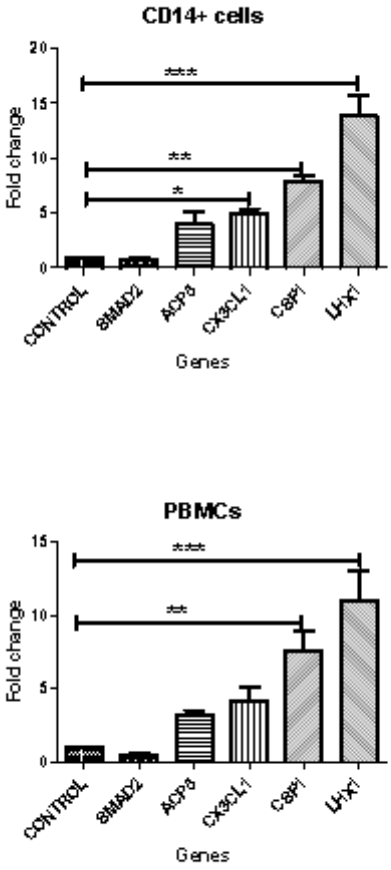

Figure 3: quantitative gene expressions of five DEGs validated using RT-PCR from- (a) CD14+ cells and (b) PBMCs. (***=significant; p-value<0.0001). All the samples were biological triplicate (Total no. N=216)

Transcription factor (TF) binding site analysis

Binding of TFs and the regulation of DEGs were analyzed by TFactS database. Altogether we found 60 different TFs of which TFs regulating only CX3CL1, CSF1, ACP5 and LHX1 were analyzed. LHX1 did not interact with any TFs. Moreover, TFs such as c-JUN, NFKB1, MITF, CREB1 etc. were involved in the pathways leading to the differentiation of osteoclast precursors to osteoclasts.

Discussion

Osteoclast precursors are progenitors of osteoclasts. Hence, determining the expression pattern of genes in osteoclast precursors may help in understanding not only the predisposition to the disease but also its prognosis. In the present study, we have analysed global gene expression pattern of osteoclast precursors for osteoporosis using microarray system. Four genes CX3CL1, CSF1, ACP5 and LHX1 were found to be differentially expressed significantly in our study samples with their involvement in bone metabolism. Up-regulation of CX3CL1 was observed in the osteoclast precursors from peripheral blood. A possible reason could be its functional involvement in the migration of osteoclast precursors from peripheral blood to the bone surface [26,27]. It is known that CSF1 through its receptor c-fms induces proliferation and survival of osteoclasts, whereas ACP5 is a well-known osteoclast specific marker [28].

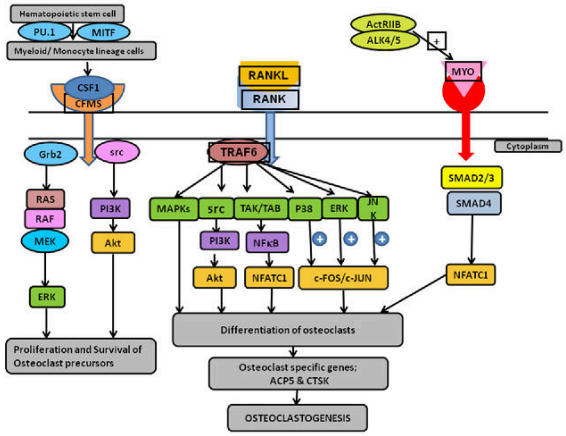

Figure 4: RAS/PI3K signaling pathway triggers downstream cascade reactions leading to osteoclast survival.

Our results of DEGs, TFs, PPIs, gene ontology and previous findings (29-34) suggest that hematopoietic cells in presence of transcription factor PU.1 form Myeloid/ Monocyte lineage cells like osteoclast precursors [29,30]. CSF1 on binding with its receptor c-fms on osteoclast precursor provides signals for osteoclast proliferation which are manifested through RAS and PI3K dependent mechanisms [31,32]. RAS a small G-protein promotes activation of RAS/PI3K and Raf/MEK/ERK pathway [33]. RAS/PI3K signaling pathway triggers downstream cascade reactions leading to osteoclast survival (Figure4).

In the RAS signaling pathway, Angiotensin II type 1 receptor (AT1) induces osteoclast differentiation by decreasing osteocalcin activity and induces high bone turnover leading to osteoporosis [34]. On the other hand, PI3K activity is either regulated by RAS activation or mediated independently. On receiving the signals from CSF1, cytoplasmic tyrosine residues of c-FMA get phosphorylated along with c-src recruitment. This process induces PI3K to activate AKT and ERK signaling pathways resulting into the proliferation and survival of osteoclast precursors [35-38]. Further, PI3K-RAP1 signaling pathway actively promotes osteoclast cell adhesion through extracellular ADP factor and mediates focal adhesion turnover assembly [39]. Our results based on pathway analysis of DEGs were in accordance with these earlier findings.

Similarly, the expression of RANK in early osteoclast precursors is essential for their differentiation to mature osteoclasts [40]. Binding of RANK-RANKL recruits TRAF6, which activates TGF-beta –activated Kinase 1 (TAK1) and downstream signaling factors such as P38 mitogen-activated protein kinase (P38 MAPK), NF-κ-beta (p50/52)

and JNK1 inducing c-FOS/c-JUN. NF-κ-beta in turn activates NFATC-1, one of the vital transcription factors along with MITF and CREB1 that are involved in the differentiation of osteoclast precursors to mature osteoclasts [41]. At this state osteoclast specific genes such as ACP5, CTSK, etc. exhibit increased expression. Our microarray data also show up- regulation of these TFs and genes. However, one of the reasons for low RANK expression in our microarray data could be the circulating osteoclast precursors. It has been reported that osteoclast precursors generated from circulating monocytes express low level of RANK (RANKlow and c-FMShigh) than those derived from bone marrow stromal cells [2]. Our analysis for gene ontology also supports these findings. In the present study we observed significant enrichment of TGF-beta signaling pathway process, transcription factors such as NF-κ-beta, c-JUN, CREB1, MITF, etc. and significantly high expression of ACP5 gene.

Earlier study by Trost, et.al suggested role of oxidative stress on pathogenesis of osteoporosis. Upregulated AOX1, TXNRD1, GSR and MT1G genes in osteoblast cells are important mediators of the stress response [42]. Our results on osteoclast precursors did not show differential expression of these genes suggesting increased tendency towards osteoclastogenesis. Liu, et.al in Caucasian population found three genes viz., CCR3, HDC and GCR to be significantly upregulated. These genes play an important role in monocyte recruitment into bone and their differentiation into osteoclasts [20]. In our study we did not find these markers to be upregulated significantly. This difference could be because of difference in the ethnicity and selection of monocyte subpopulation containing CD16+ cells by Liu, et.al. Whereas, our study specifically involves $CD14^+$ cells which are putative osteoclast precursors in the peripheral blood.

Apart from these bone specific genes LIM Homeobox (LHX1) gene was also up regulated in the osteoporosis group in our study. Previous studies have shown involvement of LHX1 gene in the intramembranous skull formation during development [43,44]. There are virtually no reports on the direct association of LHX1 with osteoporosis. However, LHX 2 is strongly expressed in osteoclast precursors and reported to regulate osteoclastogenesis by modulating RANKL signals. LHX 2 knockout mice exhibited osteoporotic bone phenotype with increased osteoclast formation [45]. Moreover, LHX1 is regulated by transcription factors such as ATF2, c-FOS/c-JUN, which were not only up regulated in our study but also top the list of ten transcription factors, suggesting the involvement of LHX1 in osteoporosis. Hence, we propose it as a novel gene for the predisposition for osteoporosis in the postmenopausal Asian Indians.

Conclusion

Our microarray analysis for primary osteoporosis in Asian Indians reveals a complex interaction of cytokines, genetic markers, their regulatory elements and signaling pathways, which may play a central role in elevated osteoclastogenesis . Cytokine CSF1 along with genetic markers such as ACP5, CX3CL1 endorses osteoclastogenesis through PI3k-Akt and TGF-β signaling pathways. LHX1 appears to be a novel gene implicated in osteoporosis suggesting its crucial role in pathogenesis of osteoporosis especially in the postmenopausal Indian females. This may aid in molecular pathogenesis and developing novel prospective for prognosis against primary osteoporosis for Asian Indian population.

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Authors' Contributions

RA and MW designed the study plan. MR has carried out the clinical and molecular work along with SM. PG, RA and MR has performed the microarray data analysis. MW has provided his expertise in editing the manuscript along with RA. All authors read and approved the final draft of manuscript.

Ethics Approvals

Present study was approved by ethics committee of Savitribai Phule Pune University. Biological samples were collected after obtaining written informed consent from female volunteers.

Conflict of Interest

Mehrunnisa M. Raje, Suhas T. Mhaske, Payel Ghosh, Mohan R. Wani and Richa Ashma declares that they have no conflict of interest

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