Acupuncture and Herbal Medicine

Acupuncture-induced gene co-expression networks in postmenopausal women with osteoarthritis and osteoporosis: *in-silico* analysis

Michell E. García-Espinosa[1], Paul Limias-Quezada[2], Alejandra I. Ortega-Meléndez[3], Martha A. Ballinas-Verdugo[4], Rosa E. López-Gómez[2], Emma López-Espinosa[2], Rogelio F. Jiménez-Ortega[1,2,*]

[1]Laboratorio de Genómica del Metabolismo Óseo, Instituto Nacional de Medicina Genómica (INMEGEN), Mexico City, Mexico; [2]Unidad de Acupuntura Humana Rehabilitatoria, Universidad Estatal del Valle de Ecatepec (UNEVE), Ecatepec de Morelos, Mexico; [3]Unidad de Ciencias de la Salud, Universidad ETAC campus Coacalco, San Francisco Coacalco, Mexico; [4]Laboratorio de Inmunología, Instituto Nacional de Cardiología "Ignacio Chávez," Mexico City, Mexico

Abstract

Objective: Bone is a tissue that is constantly remodeled to adjust the microarchitecture and maintain the mechanical needs of bone through the balance of bone resorption and formation processes. Alterations in these processes can lead to the development of different diseases, such as osteoarthritis and osteoporosis. In recent years, it has been shown that acupuncture is an effective treatment for pain, physical dysfunctions, and the immune system, so the stimulation of acupuncture points could affect genes associated with inflammatory processes and, therefore, osteoarthritis and osteoporosis. To analyze changes in gene expression post-acupuncture in data from a group of individuals with osteoarthritis that also manifests in osteoporosis.

Methods: Through using microarray technology and bioinformatics analysis, potential genes associated with osteoarthritis after acupuncture treatment are identified and compared with genes implicated in osteoporosis. The genes identified in each disease were evaluated through a Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analysis, where the results allowed the generation of an *in-silico* model that shows interaction networks between signaling pathways and genes involved in both diseases.

Results: In this interaction, 37 differentially expressed genes were identified in patients with osteoarthritis before and after acupuncture treatment, and 665 differentially expressed genes were involved in osteoporosis. In the osteoarthritis group, 15 signaling pathways involved in this disease were obtained, and for osteoporosis, 13 signaling pathways associated with immunological processes that participate in bone metabolism were obtained osteoarthritis and osteoporosis are two age-associated diseases that are characterized by alterations in the bone remodeling mechanism induced by changes in gene expression profiles.

Conclusions: Treatment with acupuncture can modify various cytokines involved in diseases related to the immune system so that it can have beneficial effects on osteoarthritis and osteoporosis. In addition, bioinformatics analysis allows us to know those signaling pathways through which they could have acupuncture effects.

Keywords: Acupuncture, Genes, Osteoarthritis, Osteoporosis, Signaling pathway

Graphical abstract: http://links.lww.com/AHM/A137.

Introduction

Bone is a tissue that constantly remodels to maintain the balance between bone resorption, regulated by osteoclasts, and bone formation, regulated by osteoblasts[1]. Bone remodeling consists of three consecutive phases: 1) bone resorption, where osteoclasts are chemoattracted to sites of damaged or deteriorated bone and whose function is to reabsorb this tissue; 2) the reverse phase, where mononuclear cells are attracted to the surface of the bone; and 3) formation when osteoblasts form new bone until it is completely replaced[2]. This mechanism is responsible for adjusting bone microarchitecture and maintaining constantly changing mechanical needs, as well as repairing damage to the bone matrix and preventing bone tissue deterioration[3]. Some primary regulators of bone remodeling are parathyroid hormone (PTH), calcitriol, sex hormones, insulin-like growth factor (IGF), bone morphogenic protein (BMP), growth hormone, thyroid hormones, sex hormones, and a host of cytokines and growth factors that affect bone cell

Michell E. García-Espinosa and Paul Limias-Quezada contributed equally to this work and shared first authorship.

*Corresponding author. Rogelio F. Jiménez-Ortega, E-mail: rogeliofrank.jimenez@uneve.edu.mx.

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functions both systemically and locally[4]. Furthermore, through the receptor activator nuclear kappa B (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG) system, the processes of bone resorption and formation are closely coupled in such a way that after a cycle of resorption, a cycle continues of bone formation to maintain the integrity of the skeleton[5]. Alterations in bone remodeling such as mechanical stress, trauma events, autoimmune factors, aging, menopause, and hormonal alterations can cause pathological changes, generating aberrant bone turnover and consequently the development of bone diseases[6]. The increase in bone remodeling activity, the presence of sclerosis, bone marrow lesions, and the increase in the vascularization of the subchondral bone are distinctive characteristics of osteoarthritis (OA)[7], while the deterioration in microarchitecture and decreased mass bone leading to reduced bone strength and resistance are hallmark features of osteoporosis (OP)[8]. OA is a chronic, degenerative, and progressive joint disease characterized by affecting the articular cartilage, subchondral bone, ligaments, and synovial membrane. This disease involves underlying inflammatory processes that are responsible for regulating its development and progression. It is the result of traumatic injuries to joints, such as injuries to soft tissues, chondral surfaces, ligaments, tendons, menisci, fractures, or even surgical interventions in the joints[9]. Some of the factors that influence radiological incidence have been identified through epidemiological studies, including obesity, heredity, presence of OA in the hand, strength in the quadriceps, imbalance in hormonal profiles, and gender (mainly women postmenopausal)[10]. However, the effect of these factors on articular cartilage is not entirely clear, so studies are necessary to understand the mechanisms involved in the development of OA This disease was associated with aging, in 2020, when it was reported to affect approximately 595 million people, which is equivalent to 7.6% of the world population, of which adults over 70 years of age are the most affected[11]. On the other hand, OP is a disease characterized by loss of bone mass and deterioration of microarchitecture, leading to increased bone fragility and susceptibility to fracture. This disease affects approximately 200 million people in the world, mainly women over 50 years of age. It is responsible for more than 8.9 million fractures per year, the most frequent being the hip, spine, distal forearm, and proximal humerus. This disease is responsible for morbidity and mortality in patients and has a high impact on healthcare systems. The economic, medical, and social burden of OP fractures remains high even in developed countries and is currently constantly increasing in other countries around the world[12–14]. Currently, OA and OP are considered degenerative diseases, and there is controversy as to whether they are related to each other[15]. Some studies have focused on understanding the prevalence of OP in postmenopausal women with primary knee OA in the Indian population, which is characterized by generalized vitamin D deficiency[16]. In a 10-year follow-up study, it was observed that OP in the lumbar spine could reduce the risk of developing OA. On the contrary, the authors mention that hypertrophic osteophytes in lumbar OA can reduce the incidence of lumbar fractures. However, the study does not indicate whether the OA status was due to the effect of OP progression[17]. Additionally, it has been mentioned that knowledge about the convergent and divergent risk factors between OA and OP has given rise to new conclusions about the role of bone mineral density (BMD), body mass index (BMI), falls, and genetics concerning disease pathophysiology and increased fracture risk in OA and OP[18]. Currently, there are different drugs authorized to reduce the risk of fractures that are aimed at slowing bone resorption (bisphosphonates) or stimulating bone formation (teriparatide). However, some drugs have had adverse effects that imply an increased risk of cardiovascular events, thromboembolic disease, and breast cancer, as well as rare but potentially severe side effects[19]. Therefore, in recent years, new treatments have been suggested that can increase BMD and prevent fractures. In this regard, many studies have reported that acupuncture is an effective treatment for different diseases, including OA and OP. However, there is little information on its physiological effect, mainly on changes in mRNA expression levels after acupuncture treatment. A widely used tool in personalized medicine that has helped to identify new molecules used in early detection and the identification of new therapeutic targets is bioinformatics. This tool has made it possible to analyze data from technologies for whole genome analysis, including microarrays and next-generation sequencing (NGS), through which mutations or genetic variants can be identified with a high level of sensitivity, as well as changes in the expression profiles of genes involved in different pathologies or induced by acupuncture treatment[20]. Bioinformatics analyses can provide a basis for the validation of functional regulators in OA/OP for the construction of models that convey a molecular vision of the mechanisms and a comprehensive framework to discover new pharmacological targets and biomarkers used in the diagnosis of bone diseases, prognosis, and monitoring of acupuncture treatment. Therefore, in this work, through an *in-silico* analysis, we seek to evaluate the changes in gene expression induced by acupuncture in individuals with OA and OP and understand the functional role of candidate genes and the signaling pathways in which they are involved[21].

Material and methods

Data acquisition and preprocessing

In this work, a systematic review of the literature was carried out. Studies aimed at identifying changes in the genetic expression of OA/OP influenced by age and whose data were available in public databases were searched. Expression data were selected based on the following inclusion criteria: Those studies that analyzed changes in the gene expression of patients with OA and OP, that have used acupuncture aimed at the treatment of OA/OP and that used platforms for whole genome analysis such as expression microarrays, NGS and/or TaqMan low-density arrays (TLDAs).

Expression analysis of microarray data

To analyze changes in the expression profiles of genes involved in OA, data from the GeneChip Human Genome U133 plus 2.0 microarray (Affymetrix, Thermo

Fisher Scientific, Inc., Santa Clara, CA, USA) were analyzed. Files in CEL format were obtained from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) with accession number GSE59526. The authors of this study analyzed samples from patients with a confirmed diagnosis of OA through X-rays. As inclusion criteria, they took into account those individuals who were in the active stage of the disease, were not resistant to Methotrexate (MTX), Leflunomide, negative for pregnancy, positive for anti-cyclic citrullinated protein antibodies (anti-CCP) and who agreed to sign an informed consent. In contrast, in the exclusion criteria, the diseases related to the hematological, endocrine, and nervous system were not selected. Also, the participants medicated with MTX, leflunomide, cortical hormone, tumor necrosis factor (TNF)-α antagonists, interleukin (IL)-6, CD20 mono-antibody, immunosuppressants, penicillamine or chloroquine were excluded. The details of the selection can be consulted in NCBI (https://clinicaltrials.gov/study/NCT01619176#participation-criteria) with accession number NCT01619176. The expression profiles of nine patients from the American College of Radiology were analyzed to whom two therapies were administered, both with doses of 7.5 to 15 mg/week of methotrexate, 100 mg twice a day of the non-steroidal anti-inflammatory drug (NSAID) and 20 mg/day of leflunomide for 3 months, where the control group was three patients and the group experimental (six patients) which received the same treatment plus acupuncture through the stimulation of the Zusanli (ST36); Taixi (KI3) and Shenshu (BL23) were the basic points, and Waiguan (SJ5); Baxie (ExUE9); Yinlingquan (SP9); Quchi (LI11); Yanglingquan (GB34); Xuehai (SP10); Dazhu (BL11); Dazhui (DU14) and Pishu (BL20), details on points of stimulation (https://clinicaltrials.gov/study/NCT01619176). Each patient had a peripheral blood sample taken before and 2 weeks after acupuncture therapy in the AP group and 3 months later for both therapies. Eighteen total RNA samples were extracted (two samples from each patient) and analyzed through Affymetrix microarray technology (HGU133 plus 2.0)[22]. On the other hand, the expression data for OP were obtained from Jiménez-Ortega et al.[23]. Where a differential expression analysis of miRNAs and transcriptome was performed in 12 Mexican postmenopausal women, six with low BMD (OP) and six with high BMD (normal), using the GeneChip Human 133 plus 2.0 microarray in Affymetrix platform. For OP patients *versus* high BMD patients where the files in CEL format were obtained, the inclusion criteria included postmenopausal women equal to or greater than 12 months after the cessation of their menstruation, with spinal *T* score or hip <−2.5 standard deviations (SD), and *T* score >−1.0 SD for the group with high BMD, also, women with an age range from 63 to 85 years were considered, and all participants signed a letter of informed consent. The exclusion criteria were to have severe residuals of cerebrovascular disease, diabetes mellitus, chronic kidney disease manifested by serum creatinine >1.9 mg/dL, chronic liver diseases or alcoholism, treatment with corticosteroids, with anticonvulsant therapy or disease of any endocrine organ that could affect bone mass[23]. To assess the sample size (replicates per condition) for each microarray dataset, we used the coefficient of variation (CV) method, which is recommended by Affymetrix and is based on calculating the signal intensity percentiles (25th, 50th, and 75th percentiles) for a dataset[24]. This method compares the CVs calculated for the number of replicates for each percentile. It allows us to determine the optimal sample size when the CV value stabilizes and does not change appreciably from "n" replicates to "n" replicates. Therefore, increasing additional replicates is unlikely to significantly improve the accuracy of estimating sample standard deviations. The raw microarray data used in this study showed that the CV reaches stability in seven replicates for the 25th, 50th, and 75th percentiles of signal intensity, so our sample size is sufficient to provide statistical power. Suitable for detecting differential changes in gene expression.

Data processing and screening of differentially expressed genes

Differential expression analysis was performed from the original files in CEL format, which were processed into expression values through the robust multiarray analysis (RMA) normalization method. The analysis was conducted using the "R" software and the Affy package. This method consists of three main steps: 1) background correction, 2) normalization, and 3) summarization. The following cut-off criteria established the choice of the under-regulated and over-regulated genes: They presented a change rate >0.5 and <−0.5, respectively, as well as a false discovery rate (FDR) value <0.05[25]. The method to identify differentially expressed genes (DEGs) is performed through the evaluation of the logarithmic ratio between two conditions, and all genes that differ by more than an arbitrary cut-off value are considered differentially expressed. Suppose the cut-off value chosen is a two-fold difference. In that case, genes are considered to be differentially expressed if the expression under one condition is more than two-fold higher or lower than under the other condition. This test is known as "Fold"; it is not a statistical test, and there is no associated value that indicates the level of confidence in the designation of genes as differentially under- or overexpressed. The "Fold" value is subject to bias if the data have not been properly normalized. Finally, the Student *t* test determines which genes are differentially expressed[26].

Selection of potential candidate genes

The overexpressed or underexpressed genes derived from the HGU133 plus 2.0 Affymetrix microarray analysis of the OA and OP groups were analyzed through a comparative analysis (Venn diagram). This analysis allowed us to know those genes whose expression profile has been influenced by general acupuncture and is shared between OA and OP. The set of genes shared between both conditions was analyzed using the STRING/Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway database (https://string-db.org/) and the ShinyGO v0.741 Enrichment analysis + more software (http://bioinformatics.sdstate.edu/go74/) of Homo sapiens to obtain and select those signaling pathways involved in alterations in bone metabolism and to obtain enrichment pathways that show the magnitude between

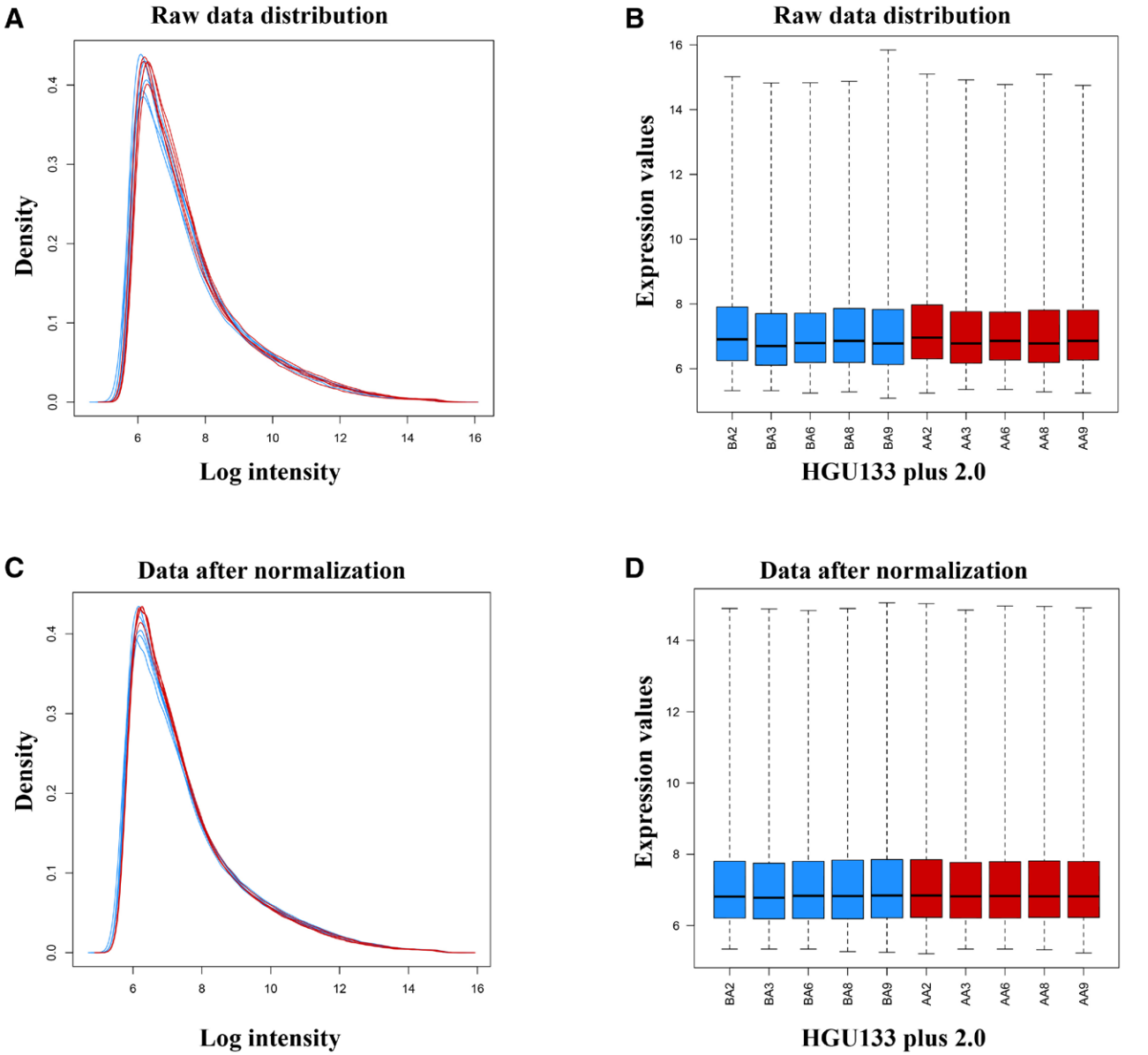


Figure 1. Distribution data of the microarrays, the data set is shown in blue before acupuncture treatment and in red after acupuncture treatment. (A) Intensity histogram, a slight variation is observed between the density of the probes for each microarray of the analyzed samples. (B) Boxplot, the distribution and variation between the medians of each microarray is observed. (C) Intensity histogram showing a significant decrease in the density of probes after normalization. (D) Boxplot, a decrease in the variability of the medians of each microarray is observed. AA: After acupuncture; BA: Before acupuncture.

the enrichment pathways and allow to generate an interaction network between the most significant biological processes. The selection of genes involved in OA and OP was also used to develop an interaction network between genes involved in signaling pathways using Cytoscape v3.7.2 software.

Genes associated with different diseases

To know the association of potentially related genes between the different OA/OP signaling pathways, as well as their involvement with different diseases, the DAVID Functional annotation bioinformatics microarray analysis database was used (https://david.ncifcrf.gov/) and the GAD_Disease tool, taking into account the most significant diseases according to the Cohen coefficient (Kappa), which calculates the effect of chance on the proportion of observed agreement[27].

Results

Data acquisition and preprocessing

Through HGU133plus 2.0 microarray processing, a total of 54,675 genes were obtained per 10 samples. The data set from all conditions was processed into expression estimates through the RMA method as shown in Figure 1. The medians of each microarray show a high level of variability concerning the data after normalization which demonstrates a high level of standardization according to their similarity score.

Expression microarray analysis

The matrix of DEGs from the microarray analysis of the OA after acupuncture treatment group is shown in Supplementary Table 1 (http://links.lww.com/AHM/A147) and the list of DEGs of the OP group is shown

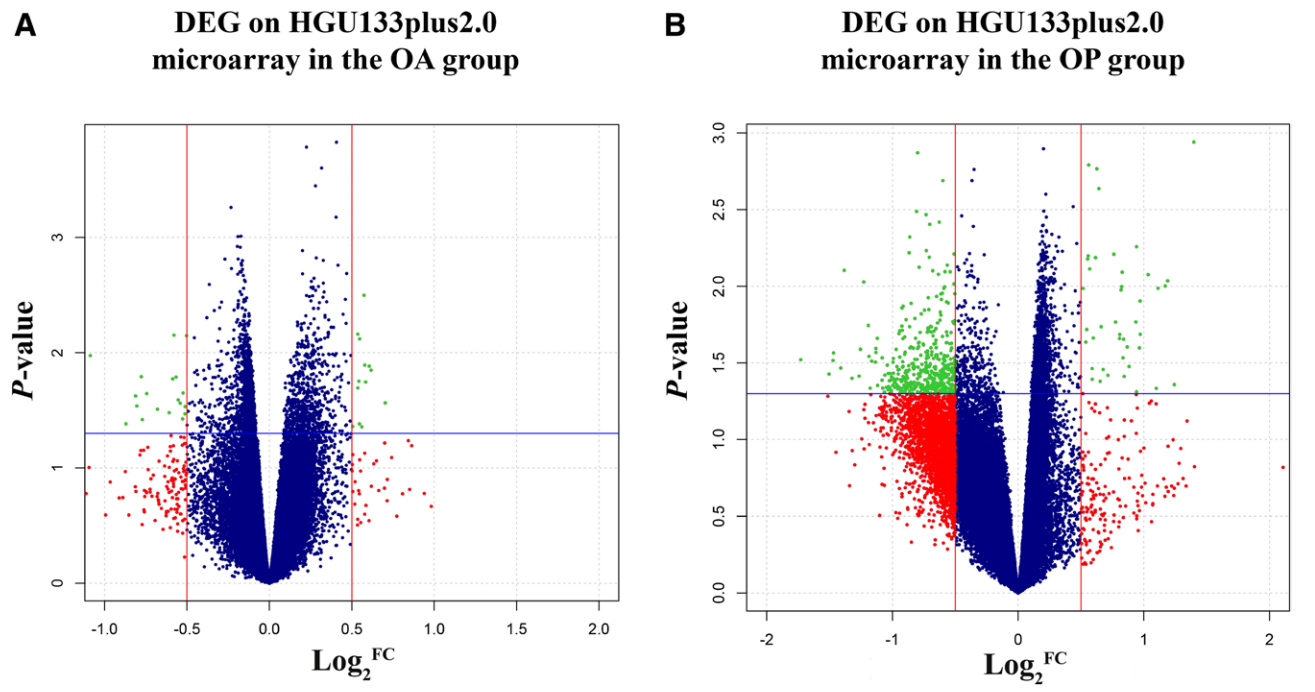


Figure 2. Volcano plot. (A) The DEG from the microarray analysis of the group of individuals with OA is observed after treatment with acupuncture. (B) The DEG from the microarray analysis of the group of individuals with OP is observed. The DEG are shown in green according to the established cut-off criteria, underexpressed (<–0.5 fold change) and overexpressed genes (>0.5 fold change) and *P* value <0.05 ($-\text{Log}_{10}$). DEG: Differentially expressed genes. OA:osteoarthritis,OP:osteoporosis.

in Supplementary Table 2 (http://links.lww.com/AHM/A148). For the OA patient group after the acupuncture treatment, a total of 37 DEG were identified which met the previously established cut-off criteria (fold change <–0.5 and >0.5 and a *P* value <0.05). A total of 22 underexpressed genes and 14 overexpressed genes were identified (Figure 2A). On the other hand, for the group of patients with OP, a total of 665 DEG were identified, of which 614 were underexpressed and 51 were overexpressed according to the cut-off criteria (Figure 2B).

Associated gene enrichment analysis

To explore the possible functional association between DEGss, a signaling pathway analysis was performed through the KEGG pathway bioinformatics tool and ShinyGO v0.741 in homo sapiens for OA and OP data, respectively[28]. For the OA group, the analysis revealed 15 signaling pathways involved in metabolic processes (Table 1), which are represented through an interaction network between signaling pathways (Figure 3). Subsequently, the same procedure was performed in the OP group where 40 signaling pathways were observed, of which 13 are associated with OP (Table 2). The interaction between the signaling pathways is shown in Figure 4.

Genes associated with different diseases

To know the functional role of genes associated with bone metabolism present in OA and OP, the Venn diagram made between the genes from the analysis of signaling pathways of the OA and OP group shows 13 genes exclusive to OA, 81 genes exclusive to OP, and 8 genes shared between both signaling pathway analyzes were observed (Figure 5A). The rate of change in expression of the genes co-expressed between both pathologies, their statistical significance, their position within the genome, and the function they can play in bone metabolism is shown in Table 3[29–36].

Subsequently, in the enrichment analysis carried out with DAVID and the GAD_Disease tool to know the association of the selected genes with different diseases, it was observed that they are mainly involved with rheumatoid arthritis, atherosclerosis, and lymphocytic leukemia (Figure 5B). Finally, the interaction network generated between genes associated with OA/OP is shown, where the interaction of the eight genes with different signaling pathways can be observed (Figure 5C).

Discussion

OA and OP are two of the most common skeletal diseases associated with aging. Their relationship is controversial since different cross-sectional studies have reported that OA is associated with an increase in BMD[37]. On the other hand, OP has also been reported to be a disease that promotes the development of OA. A meta-analysis study reported that the worldwide incidence increases with aging, promoting the development of OA through different mechanisms[38], in another prospective study it was reported that elderly participants with radiographic OA of the hip and knee showed an increase in total hip bone loss[39], while in a rabbit model, it was observed that the development of OP aggravates cartilage lesions due to an increase in fragility and loss of quality of the subchondral bone in addition to the activation of bone remodeling[40–41]. Furthermore, in studies that used animal models with early OA, it was observed that they showed an increase in the rate of bone turnover, while in

Table 1

Signaling pathways associated with osteoarthritis after acupuncture treatment

Pathways	Number of genes	Genes	Pathway genes	Fold enrichment	Enrichment FDR
Negative regulation of macromolecule metabolic process	9	*DNAJA1, OAS1, CBX5, PCIF1, BHLHE40, DUSP6, RPL10, PER1, H1-10*	3,253	3.00	0.049
Negative regulation of nitrogen compound metabolic process	8	*DNAJA1, CBX5, PCIF1, BHLHE40, DUSP6, RPL10, PER1, H1-10*	2,619	3.31	0.049
Dephosphorylation	4	*CDC14A, PCIF1, DUSP6, DUSP2*	551	7.88	0.049
Peptidyl-tyrosine dephosphorylation	3	*CDC14A, DUSP6, DUSP2*	116	28.07	0.038
Negative regulation of MAPK cascade	3	*DNAJA1, DUSP6, PER1*	162	20.10	0.038
Regulation of stress-activated MAPK cascade	3	*DNAJA1, PER1, RELL1*	191	17.05	0.045
Regulation of stress-activated protein kinase signaling cascade	3	*DNAJA1, PER1, RELL1*	194	16.78	0.045
Stress-activated protein kinase signaling cascade	3	*DNAJA1, PER1, RELL1*	268	12.15	0.049
Stress-activated MAPK cascade	3	*DNAJA1, PER1, RELL1*	251	12.95	0.049
Entrainment of circadian clock	2	*BHLHE40, PER1*	34	63.85	0.038
Response to interferon-beta	2	*OAS1, AIM2*	31	70.03	0.038
Cellular response to interferon-beta	2	*OAS1, AIM2*	23	94.39	0.038
Peptidyl-threonine dephosphorylation	2	*DUSP6, DUSP2*	25	86.84	0.038
Negative regulation of JNK cascade	2	*DNAJA1, PER1*	38	57.13	0.043
Negative regulation of stress-activated MAPK cascade	2	*DNAJA1, PER1*	48	45.23	0.045

AIM2: Absent in melanoma 2; *BHLHE40*: Basic helix-loop-helix family member E40; *CBX5*: Chromobox 5; *CDC14A*: Cell division cycle 14A; *DNAJA1*: DnaJ Heat Shock Protein Family (Hsp40) Member A1; *DUSP2*: Dual-specificity phosphatase 2; *DUSP6*: Dual-specificity phosphatase 6; FDR: False discovery rate; *H1-10*: H1.10 linker histone; *OAS1*: Oligoadenylate synthetase 1; *PCIF1*: Phosphorylated CTD interacting factor 1; *PER1*: Period circadian regulator 1; *RPL10*: Ribosomal protein L10; *RELL1*: RELT-like 1.

proximal tibia samples from human corpses that developed early OA, the elastic modulus of the subchondral bone was observed to decrease independently of the increase in bone volume[42]. This bone softening may be accompanied by a decrease in BMD, which may be due to inadequate mineralization caused by an increase in bone remodeling activity[43]. On the other hand, OP is a recurrent disease in the aging population, it has been shown that pro-inflammatory cytokines play a key role in the pathogenesis of this disease, due to positively regulating OP and participating in the process of bone resorption or formation[44–45]. Some proinflammatory cytokines influence bone remodeling. Bone-forming proteins such as osteopontin (OPN) and osteocalcin (OCN) decrease in OP, while proteins associated with resorption increase. These include tartrate-resistant acid phosphatase (TRAP), charybdotoxin (CTX)-I, TNF-α, IL-1, and IL-6, leading to a decrease in femur BMD and bone mineral content[46]. In a study conducted by Gao et al.[47], in mice, rheumatoid arthritis was shown to induce bone mass loss and deterioration of bone quality with a high level of collagen-induced turnover (CIA), CIA rats showed high levels of IL-6, TNF-α in serum. One of the most important factors in the pathogenesis of OA/OP is an imbalance in cytokines, which leads to end effects such as damage to cartilage and other inter-articular structures through the activation of catabolic enzymes such as matrix metalloproteinases (MMP) and important inflammatory mediators such as IL-1, IL-6 and TNF-α which are activators of a large number of signaling pathways that in turn activate other cytokines and pathological processes[48]. Part of this process is chemokines which are stimulated by chemoattracting cytokines from inflammatory cells to the joint further promoting the secretion of inflammatory factors and disease progression[49]. Evidence suggests that OP may be associated with an increased risk of developing OA, indicating that measures to raise BMD may be effective in preventing OA and this is reflected in the role played by the eight genes shared between OA/OP that were identified in this analysis (Figure 5A), for example, *CDKN1C* gene encodes a protein inhibitor of several cyclin G1 complexes and a negative regulator of cell proliferation, indicating that underexpression of this gene could act in response to harmful mechanical stress and promote OA[50]. However, its potential role associated with the development of OP has not been reported. *FNIP2* gene encodes a tumor suppressor folliculin and AMP-activated protein kinase (AMPK), which is an

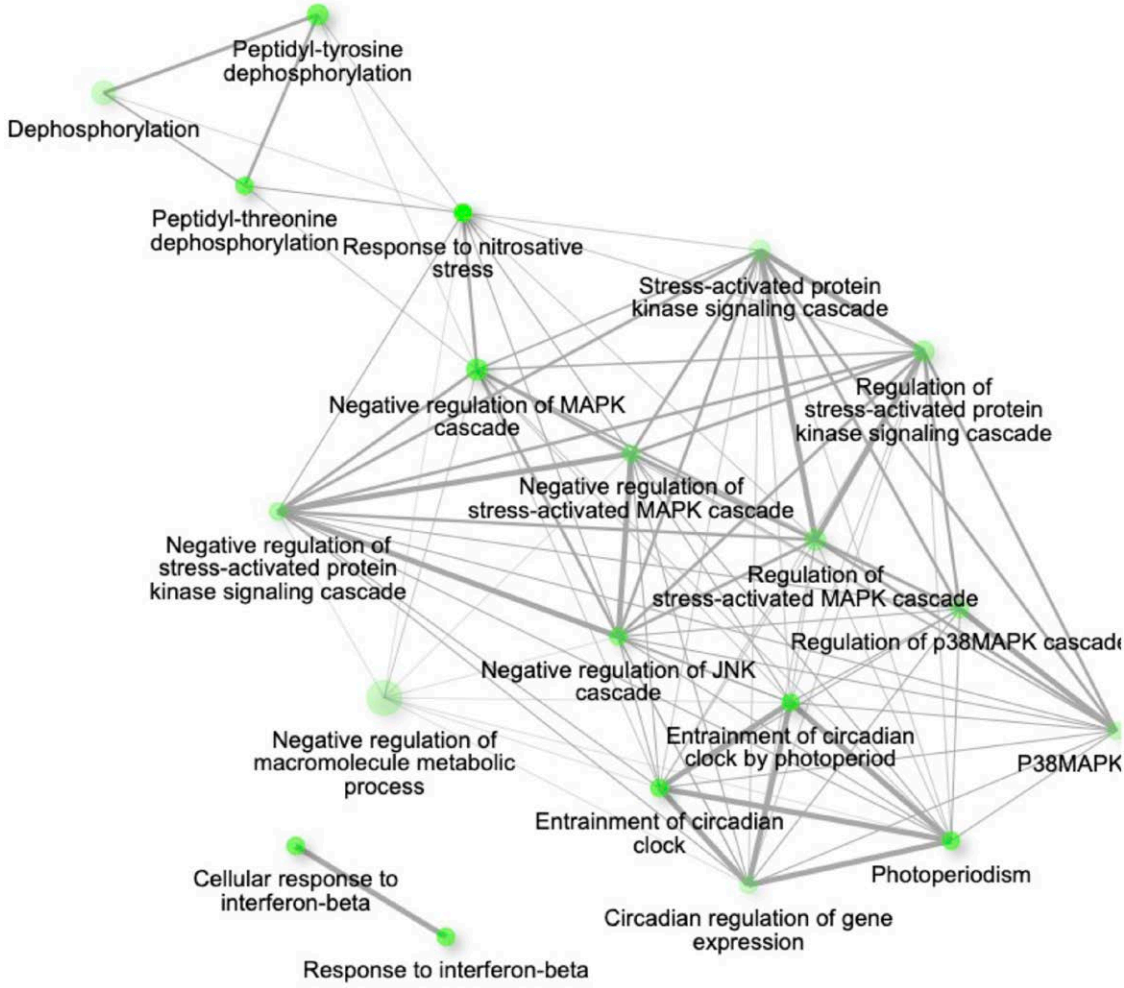


Figure 3. Signaling pathway associated with OA. The interaction between signaling pathways related to biological processes involved in OA is shown. The gray lines represent the interaction between pathways, which are represented by green nodes. Those pathways with greater interaction represent greater participation in bone metabolism pathways whose deregulation could lead to the development of OA. MAPK: Mitogen-activated protein kinases; OA: Osteoarthritis.

energy sensor that has been described for its role as an inhibitor of osteoclastogenesis. Therefore, it is possible to deregulate to *FNIP2* and promote osteoclastogenesis in patients with OP[51]. In OA, the function of the AMPK protein is decreased, which is associated with reduced autophagy. This leads to impaired mitochondrial function, the formation of high levels of reactive oxygen species, and inflammation of joint tissue. In addition, other abnormalities, such as synovial inflammation and abnormal remodeling of subchondral bone, trigger joint tissue degeneration[52]. The protein encoded by *FGD2* gene belongs to a family of guanine nucleotide exchange factors (GEF) that control rearrangements. Cytoskeleton-dependent membrane cells activating the CDC42 protein. *FGD2* gene is expressed in B lymphocytes, macrophages, and dendritic cells. It has been reported that its deregulation in osteoclast precursors could promote cell proliferation and differentiation[53]. *ITPRIPL2* gene encodes a protein that is predicted to be an integral component of the membrane of mesenchymal stem cells, so its deregulation could be associated with alterations in the differentiation of osteoblasts and chondrocytes[54]. KMO gene encodes a protein located in the outer membrane of the mitochondria. Its function is to catalyze the hydroxylation of the metabolites L-tryptophan and L-kynurenine to form L-3-hydroxykynurenine. KMO protein also carries out the synthesis of quinolinic acid, which affects NMDA-induced signaling in pancreatic beta cells, myocardial cells, the gastrointestinal tract, and osteoblasts[55]. On the other hand, it is reported that the overexpression of *KMO* could be involved in chondrogenesis, so its deregulation could be involved in the development of OA, but more studies are necessary to elucidate this mechanism[56]. *SAMD9L* gene encodes a cytoplasmic protein that has the function of tumor suppressor and participates in the cell proliferation mechanism. Deregulation of this gene causes alterations in the functions of cells of hematopoietic origin, such as monocytes[31]. Expression of *SAMD9L* gene in the synovial membrane has been reported in OA. This gene has been suggested as a marker of synoviocytes, which are fibroblast-like cells of the synovial membrane and play a crucial role in joint physiology and disease[57]. *SNHG9* gene encodes a protein that regulates RNA and is associated with the long non coding RNA (lncRNA) class. The overexpression of this gene is involved in the reduction of chondrocyte apoptosis in individuals with OA[34]. *SPTLC2* gene encodes an essential

Table 2

Signaling pathways associated with osteoporosis

Pathways	Number of genes	Genes	Pathway genes	Fold enrichment	Enrichment FDR
Regulation of actin cytoskeleton	14	*EZR, CRKL, ARHGEF7, NCKAP1L, ARHGEF6, KRAS, IQGAP1, VAV1, DIAPH2, TIAM1, WASF2, PIK3CD, PAK2, NRAS*	217	2.90	0.005
NOD-like receptor signaling pathway	13	*HSP90AB1, CARD8, STAT1, GBP1, ITPR2, IFNAR1, GBP2, IFI16, CAMP, STAT2, TXNIP, IRF7, DEFA4*	180	3.24	0.004
Autophagy	12	*LAMP2, ATG2B, SH3GLB1, MTMR4, PPP2CA, KRAS, EIF2S1, CTSL, PDPK1, PIK3CD, NRAS, PTEN*	141	3.82	0.003
Ubiquitin-mediated proteolysis	11	*CDC27, UBE2K, UBE2D3, FANCL, TRIP12, UBE2Z, CUL5, UBE2C, UBE2H, KLHL9, UBE2J1*	141	3.50	0.005
Growth hormone synthesis, secretion, and action	10	*CRKL, EP300, GNAI2, STAT1, ITPR2, STAT5A, KRAS, PIK3CD, CREB3L2, NRAS*	119	3.77	0.005
FoxO signaling pathway	10	*EP300, SOD2, KRAS, CCNG2, PDPK1, BCL2L11, PIK3CD, USP7, NRAS, PTEN*	131	3.43	0.007
Apoptosis	10	*BAX, ITPR2, KRAS, EIF2S1, CTSL, BCL2A1, PDPK1, BCL2L11, PIK3CD, NRAS*	135	3.33	0.008
mTOR signaling pathway	10	*KRAS, CAB39, PDPK1, ATP6V1C1, MIOS, PIK3CD, FZD2, NRAS, FNIP1, PTEN*	154	2.91	0.015
Nucleocytoplasmic transport	9	*TNPO1, KPNB1, TMEM33, IPO8, NXF3, MAGOH, XPO6, ALYREF, KPNA4*	108	3.74	0.007
Sphingolipid signaling pathway	9	*BAX, PPP2CA, GNAI2, KRAS, PDPK1, PIK3CD, CERS6, NRAS, PTEN*	119	3.40	0.010
Signaling pathways regulating pluripotency of stem cells	9	*JARID2, KAT6A, KRAS, CTNNB1, SMAD1, PIK3CD, INHBC, FZD2, NRAS*	143	2.82	0.025
B cell receptor signaling pathway	8	*KRAS, VAV1, NFKBIE, PIK3AP1, SYK, PIK3CD, NRAS, INPP5D*	81	4.44	0.006
Fc epsilon RI signaling pathway	7	*KRAS, PDPK1, VAV1, SYK, PIK3CD, NRAS, INPP5D*	68	4.62	0.008

ALYREF: Aly/REF export factor; *ARHGEF6*: Rac/Cdc42 guanine nucleotide exchange factor 6; *ARHGEF7*: Rho guanine nucleotide exchange factor 7; *ATG2B*: Autophagy-related 2B; *ATP6V1C1*: ATPase H + transporting V1 subunit C1; *BAX*: BCL2-associated X, apoptosis regulator; *BCL2A1*: BCL2-related protein A1; *BCL2L11*: BCL2 like 11; *CAB39*: Calcium binding protein 39; *CAMP*: Cathelicidin antimicrobial peptide; *CARD8*: caspase recruitment domain family member 8; *CCNG2*: Cyclin G2; *CDC27*: Cell division cycle 27; *CERS6*: Ceramide synthase 6; *CREB3L2*: CAMP responsive element binding protein 3 like 2; *CRKL*: CRK like proto-oncogene, adaptor protein; *CTSL*: Cathepsin L; *CTNNB1*: Catenin beta 1; *CUL5*: Cullin 5; *DEFA4*: Defensin alpha 4; *DIAPH2*: Diaphanous-related formin 2; *EIF2S1*: Eukaryotic translation initiation factor 2 subunit alpha; *EP300*: E1A binding protein P300; *EZR*: Ezrin; *FANCL*: FA complementation group L; *FDR*: False discovery rate; *FNIP1*: Folliculin interacting protein 1; *FZD2*: Frizzled class receptor 2; *GBP1*: Guanylate binding protein 1; *GBP2*: Guanylate binding protein 2; *GNAI2*: G protein subunit alpha I2; *HSP90AB1*: Heat shock protein 90 alpha family class B member 1; *IFI16*: Interferon gamma inducible protein 16; *IFNAR1*: Interferon alpha and beta receptor subunit 1; *INHBC*: Inhibin subunit beta C; *INPP5D*: Inositol polyphosphate-5-phosphatase D; *IPO8*: Importin 8; *IQGAP1*: IQ motif containing GTPase activating protein 1; *IRF7*: Interferon regulatory factor 7; *ITPR2*: Inositol 1,4,5-trisphosphate receptor type 2; *JARID2*: Jumonji and AT-rich interaction domain containing 2; *KAT6A*: Lysine acetyltransferase 6A; *KLHL9*: Kelch like family member 9; *KPNA4*: Karyopherin subunit alpha 4; *KPNB1*: Karyopherin subunit beta 1; *KRAS*: KRAS proto-oncogene, GTPase; *LAMP2*: Lysosomal-associated membrane protein 2; *MAGOH*: Mago homolog, exon junction complex subunit; *MIOS*: Meiosis regulator for oocyte development; *MTMR4*: Myotubularin-related protein 4; *NCKAP1L*: NCK-associated protein 1 like; *NFKBIE*: NFKB inhibitor epsilon; *NRAS*: NRAS proto-oncogene, GTPase; *NXF3*: Nuclear RNA export factor 3; *PAK2*: P21 (RAC1)-activated kinase 2; *PDPK1*: 3-Phosphoinositide-dependent protein kinase 1; *PIK3AP1*: Phosphoinositide-3-kinase adaptor protein 1; *PIK3CD*: Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta; *PPP2CA*: Protein phosphatase 2 catalytic subunit alpha; *PTEN*: Phosphatase and tensin homolog; *SH3GLB1*: SH3 domain containing GRB2 like, Endophilin B1; *SMAD1*: SMAD family member 1; *SOD2*: superoxide dismutase 2; *STAT1*: signal transducer and activator of transcription 1; *STAT2*: Signal transducer and activator of transcription 2; *STAT5A*: Signal transducer and activator of transcription 5A; *SYK*: Spleen-associated tyrosine kinase; *TIAM1*: TIAM Rac1-associated GEF 1; *TMEM33*: Transmembrane protein 33; *TNPO1*: Transportin 1; *TRIP12*: Thyroid hormone receptor interactor 12, Ubiquitin Conjugating Enzyme E2 Z; *TXNIP*: Thioredoxin interacting protein; *UBE2C*: Ubiquitin conjugating enzyme e2 C; *UBE2D3*: Ubiquitin conjugating enzyme E2 D3; *UBE2H*: Ubiquitin conjugating enzyme E2 H; *UBE2J1*: Ubiquitin conjugating enzyme E2 J1; *UBE2K*: Ubiquitin conjugating enzyme E2 K; *USP7*: Ubiquitin-specific peptidase 7; *VAV1*: Vav guanine nucleotide exchange factor 1; *WASF2*: WASP family member 2; *XPO6*: Exportin 6.

subunit of serine palmitoyltransferase, which consists of two subunits and has a crucial role in sphingolipid biosynthesis. It is responsible for catalyzing the pyridoxal-5-prime-phosphate-dependent condensation of L-serine and palmitoyl-CoA to 3-oxosphinganine. High levels of *SPTLC2* expression in human chondrocytes are involved in cell proliferation processes and collagen expression, so its deregulation could be associated with the development of OA[36]. These genes were coexpressed in both diseases and were monitored to be involved in diseases associated with the immune system. Currently with a deep understanding of the close relationship between bone and the immune system, the increasing importance of immune cells in diseases that affect bone metabolism such as postmenopausal OA/OP has been outlined[58–59]. In this analysis, we observe the effect of

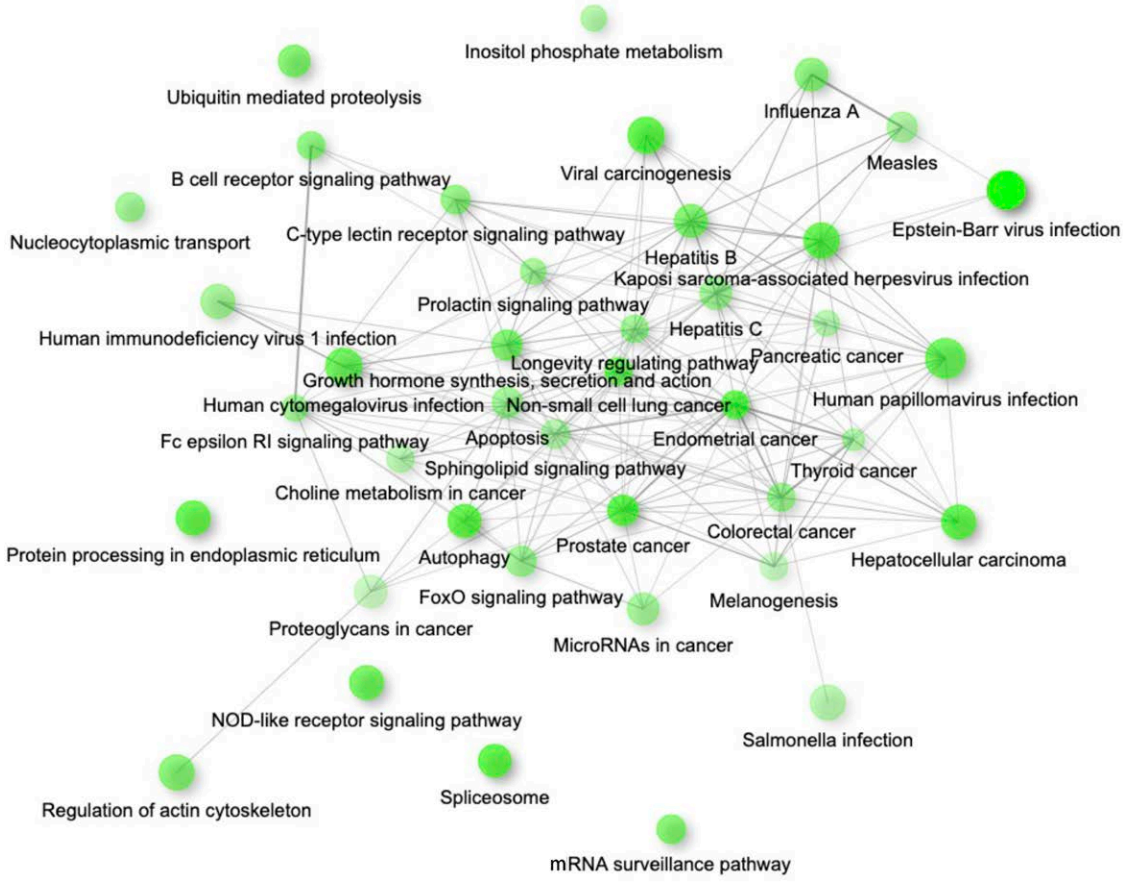
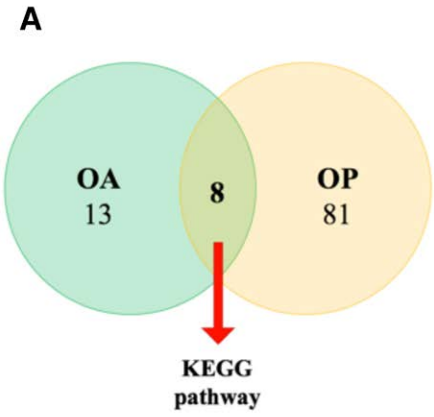


Figure 4. Signaling pathway associated with OP. The interaction between signaling pathways related to biological processes involved in OP is shown. The gray lines represent the interaction between pathways, which are represented by green nodes. Those pathways with greater interaction represent greater participation in bone metabolism pathways whose deregulation could lead to the development of OP. OP: Osteoporosis.

the immune system on different diseases that affect the bone (Figure 5B). However, more studies are required to determine the underlying processes through which OP causes OA. Additionally, in this analysis, we were able to observe the DEGs of each of the diseases analyzed, and within this set, we found the eight genes shared in both pathologies (Figure 5C). These genes have an effect on key genes that have multiple interactions, indicating that they are involved in more than one signaling pathway and their deregulation could affect different mechanisms that participate in bone metabolism.

On the other hand, acupuncture is a therapy that has its origins in traditional Chinese medicine which is focused on mitigating many diseases. It has been reported that through the stimulation of the ST36 point, anti-inflammatory, antioxidant, and immune system improvement effects are promoted[60] and it has been observed that in animal models where ST36 is stimulated with electroacupuncture, it modulates systemic inflammation induced by endotoxins, through the vagal-adrenal inflammatory axis[61]. Stimulation at Taixi (KI3), Pishu (BL20), Zusanli (ST36), and Shenshu (BL23) have been used in acupuncture treatment. Conventional therapy along with catgut implantation aimed at reducing HbA1c in addition to total cholesterol levels, low-density lipoprotein cholesterol (LDL-C), and increasing high-density lipoprotein cholesterol (HDL-C)[62]. Among the main functions of the Waiguan SJ5 point are to open the channel, eliminate stagnation, and help in the expulsion of the pathogen. This application of distal points is suggested to be used not only in the involved meridian but can also be performed in meridians of the same polarity on opposite extremities (generally *yang*). Their correspondence in general terms is: Shoulder-hip, Elbow-knee, and Wrist-ankle and they have been widely used in the treatment of OA, furthermore Baxie (ExUE9) has been applied as one of the main points in the treatment of OA on knee[63]. Yinlingquan (SP9) is responsible for increasing mean blood flow/perfusion in the spleen, but not in the liver, and has been used in the treatment of knee OA[64]. Quchi (LI11) is involved in inflammatory processes through the upregulation of IL-10 by increasing the levels of $CD3^+CD4^+$ T cells and Treg cells in mesenteric lymph nodes while downregulating the levels of $CD3^+$ $CD8^+$ T cells, Th17 cells, and TNF-α to suppress intestinal inflammation through sepsis processes[65]. Xuehai (SP10) is a point that is related to the regulation of oxidative stress and has been used in knee OA[64]. Yanglingquan (GB34) is a point that inhibits the synthesis of the Cox-2-dependent PGE2 gene, which is involved in anti-inflammatory, anti-apoptotic, and antioxidant processes. In addition, it has been reported that it can reduce the levels of the cytokines IL-1, IL-6, and TNF-α in the treatment of OA[66]. Dazhu (BL11) and Dazhui (DU14) points seek to treat cervical pain, eliminating the pathogenic factor that generates obstruction of *qi* and *blood* and toning these energies. It is mainly used in dispersion and cases of deficiency in regulation[67].



B

Num	Category	Term	Kappa
1	GAD_Disease	Arthritis, Rheumatoid	1.00
2	GAD_Disease	Atherosclerosis	0.66
3	GAD_Disease	Leukemia lymphocytic	0.55
4	GAD_Disease	Longevity	0.48
5	GAD_Disease	Infertility Male	0.39
6	GAD_Disease	Infection|Inflammation|	0.39

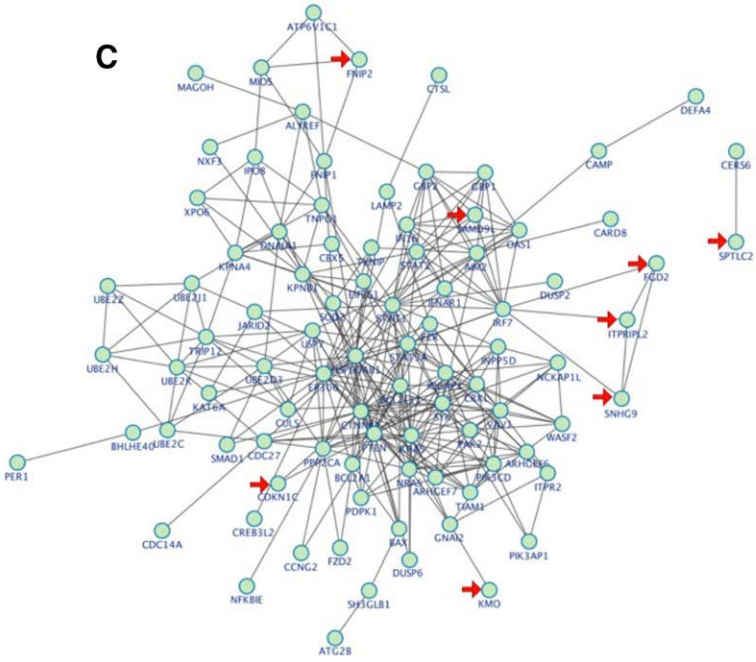


Figure 5. Signaling analysis between genes that participate in processes related to OA and OP. (A) Venn diagram between genes that participate in signaling pathways related to OA (21 genes) and OP (89), showing eight genes that interact between both diseases. (B) Generalized anxiety disorder (GAD) related to genes involved in OA and OP signaling pathways, it is observed that rheumatoid arthritis has a high level of statistical significance, followed by atherosclerosis and lymphocytic leukemia. (C) Interaction network between genes associated with biological processes of OA and OP, red arrows indicate genes shared between both bone diseases. OA: Osteoarthritis; OP: Osteoporosis.

In this sense, acupuncture has been used as a potentially useful treatment for OA since it has been used effectively for pain and physical dysfunction, so it is possible that stimulation with acupuncture can modify genes associated with inflammatory processes. Therefore, in this study, we analyzed the role of acupuncture on changes in gene expression in individuals with OA/OP and the effect it can produce on different signaling pathways associated with bone metabolism. Our results from the data processing analysis of the HGU133 plus 2.0 microarrays showed a total of 54,675 probes per 10 samples which were normalized and analyzed for their differential expression level. 37 DEG were observed that met the cut-off criteria, this number of genes is relatively compared to other microarray studies. However, it makes sense since the same individuals were analyzed three months after treatment with acupuncture and it is logical to think that their genetic expression profile did not suffer significant changes, nevertheless, the changes observed could be due to the effect of acupuncture or other effects of environmental factors such as drug consumption, diet, physical activity, alcohol consumption or exposure to different genotoxic substances during the 3 months of treatment, so through bioinformatic analysis, it was possible to elucidate the biological role of these genes and their effects on signaling pathways associated with OA/OP. On the other hand, the DEG obtained from the HGU133 plus 2.0 expression microarray of patients with OP is considerably higher, this may be because the authors analyzed changes in the differential expression profiles between a control group versus a group of individuals with OP[23]. One of the main signaling pathways identified in OA is the MAPK signaling pathway, this pathway is a member of the MAPK superfamily which is composed of four protein subtypes: P38α, P38β, P38γ, and P38δ. This signaling pathway transmits extracellular signals through a cascade reaction involving kinases that are sequentially activated to activate a variety of extracellular stimuli in articular chondrocytes and induce phosphorylation cascades. Stimuli involved in joint fluid include different inflammatory factors, cytokines, biological stress, and changes in osmotic pressure[68]. In this signaling pathway,

Table 3

Description of the genes identified as coexpressed in OA and OP

Gene ID	Name	Position	Log_2FC	*P* value	Function in bone metabolism	References
CDKN1C	Cyclin-dependent kinase Inhibitor 1C	11:2,883,213-2,885,775	–0.582↓	0.037	Involved in cell division processes and its deregulation is associated with abnormalities in the skeleton	[29]
FNIP2	Folliculin interacting protein 2	4:158,769,026-158,908,050	–0.516↓	0.054	It participates in regulating autophagic flux through modulating the FLCN–GABARAP complex, which is relevant in the development of OP	[30]
FGD2	FYVE, RhoGEF and PH domain containing 2	6:37,005,646-37,029,069	–0.777↓	0.016	Mutations in *FGD1* are associated with faciogenital dysplasia, an X-linked disorder affecting multiple skeletal structures	[31]
ITPRIPL2	ITPRIP Like 2	16:19,113,932-19,121,629	–0.586↓	0.016	Involved in alterations of methylated CpG sites shared between OA subchondral bone and overlying cartilage	[32]
KMO	Kynurenine 3-monooxygenase	1:241,532,134-241,595,642	–0.596↓	0.052	Inhibition of *KMO* inhibits the activity of enzymes at the genomic level, promotes tumor development, decreases the infiltration of macrophages and neutrophils in cardiac tissue and renal tubular necrosis. No information has been reported on bone tissue, but it could be involved in calcium metabolism	[33]
SAMD9L	Sterile alpha motif domain containing 9 like	7:93,130,056-93,148,385	–0.580↓	0.051	Gene involved in bone marrow failure that leads to the development of myelodysplastic syndrome with monosomy. Mutations in *SAMD9L* could be associated with functional alterations of the hematopoietic cells from which monocytes, precursor cells of osteoclasts, are derived	[31]
SNHG9	Small nucleolar RNA host gene 9	16:1,964,895-1,965,509	0.505↑	0.041	Overexpression of the *SNHG9* gene is associated with downregulation of miR-34, decreasing chondrocyte apoptosis in patients with OA	[34]
SPTLC2	Perine palmitoyltransferase long chain base subunit 2	14:77,505,997-77,616,637	–0.566↓	0.016	Transcription factor whose deregulation is involved in the pathogenesis of OP. Overexpression of *SPTLC2* in human chondrocytes is involved in cell proliferation and collagen expression.	[35, 36]

Upregulated = ↑. Downregulated = ↓. Log_2FC = Logaritmo base 2-fold change.

CDKN1C: Cyclin-dependent kinase inhibitor 1C; *FGD2*: FYVE, RhoGEF, and PH domain containing 2; *FNIP2:* Folliculin interacting protein 2; *ITPRIPL2:* ITPRIP like 2; *KMO:* Kynurenine 3-monooxygenase; OA: Osteoarthritis; OP: Osteoporosis; *SAMD9L:* Sterile alpha motif domain containing 9 like; *SNHG9:* Small nucleolar RNA host gene 9; *SPTLC2:* Serine palmitoyltransferase long chain base subunit 2.

participating genes include DNA homolog superfamily member 1 (DNAJA1), which encodes a heat shock protein that promotes folding, migration, aggregation prevention, and proteolytic degradation. In addition, it is considered a potential target of the bacterial DNAJ-induced immune response in rheumatoid arthritis[69]. Another gene is dual-specificity phosphatase 6 (DUSP6), which encodes a DUSP. These inhibit their target kinases through dephosphorylation of phosphoserine/threonine residues and are responsible for negatively regulating mitogen-activated protein kinases (MAP) (MAPK/ERK, SAPK/JNK, p38), which are associated with cell proliferation and differentiation. Several studies have shown that *DUSP6* regulates the severity of arthritis and joint damage because it can modulate the invasiveness of fibroblast-like synoviocyte (FLS) cells and regulate the production of cytokines such as IL-10 by Tr1 cells and the suppression of IL-6[70] so like circadian regulatory period 1 (PER1), which is a member of the "Period" family and is expressed in a circadian pattern in the suprachiasmatic nucleus, the central circadian pacemaker in the mammalian brain. In OA, the protein produced by this gene is a transcription factor that regulates its transcription and that of other genes known as clock-controlled genes, and it is thought that it may be associated with the regulation of sleep in individuals who frequently suffer from acute pain during at night and that decreases during the morning[71]. *JNK1*, *JNK2*, and *JNK3* genes are involved in the JNK signaling pathway, which interacts with the MAPK signaling pathway. JNK pathway is activated by growth factors and proinflammatory cytokines, including TNF-α and IL-1, in addition to exposure to a variety of extracellular stimuli such as ultraviolet radiation, heat, and osmotic shock[72]. The mechanism that induces cartilage alteration or degradation in OA is currently unknown, so it is thought to be a consequence of changes in chondrocytes that contribute to the destruction of joint tissue in OA[73]. Some studies suggest that activation of the JNK pathway is associated with the pathogenesis and progression of human OA. It has been reported that the interaction of JNK and ERK pathways activates c-Jun, a key component of AP-1, inducing a decrease in proteoglycan synthesis and an increase in MMP13, which is responsible for cartilage degradation. Evidence indicates that overexpression of MMP13 by chondrocytes plays a central role in cartilage degeneration[74]. On the other hand, the most important signaling pathways involved in the development of this disease are the cytoskeletal actin regulation pathway, which participates in the formation and resorption of osteoclasts. Through the formation of

a dynamic actin ring, the microfilaments bind to the vacuolar H+-ATPase (V-ATPase) and join in the formation of the wavy plasma membrane rich in V-ATPase. This interaction is necessary for the formation of wavy membranes, and its inhibition has been associated with the blocking of bone resorption in animal models with postmenopausal OP[75]. Another signaling pathway widely reported in OP is the FoxO signaling pathway, this pathway is associated with a decrease in the number of osteoblasts and increased levels of oxidative stress in these cells. *FoxO* family is made up of transcription factors that are responsible for maintaining skeletal integrity. *FoxO1* regulation of osteoblast proliferation occurs due to its interaction with *ATF4*, a transcription factor that regulates the import of amino acids; and its regulation of a stress-dependent pathway that influences p53 signaling. Consequently, decreasing oxidative stress levels or increasing protein intake normalizes bone formation and mass in mice lacking *FoxO1* only in osteoblasts. These results identify *FoxO1* as a crucial regulator of osteoblast physiology and provide a direct mechanistic link between oxidative stress and the regulation of bone remodeling[76]. Another signaling pathway that integrates protein kinases that control cellular processes is the target of rapamycin (mTOR) pathway. Genetic studies in murine models have reported that this pathway is involved in regulating multiple aspects of skeletal development and homeostasis. In addition, mTORC1 has been described as an effector that regulates the anabolic effect on bone, and its deregulation may contribute to the development of several skeletal diseases, including OA and OP[77]. Finally, in this work, we found that eight genes are shared between OA and OP, which were analyzed through the DAVID and GAD_Disease tools, finding that these are involved in the development of Rheumatoid Arthritis and other diseases associated with the immune system. Furthermore, interaction network analysis showed that these genes play a key role among different signaling pathways and their deregulation could affect multiple cellular processes, leading to the development of diseases associated with bone metabolism including OA and OP.

Conclusions

OA and OP are two diseases that affect the skeletal system and are age-related mainly in postmenopausal women. Both diseases share alterations in the bone remodeling mechanism where genetic expression profiles can be altered. Various immunological mechanisms are essential in bone maintenance processes and their alteration can lead to the development of various diseases, including those that affect bone metabolism. In recent years, it has been shown that acupuncture treatment can modify various cytokines involved in diseases related to the immune system including OA and OP. Our results showed that acupuncture treatment has an important role in changes in the genetic profiles of OA patients that affect different signaling pathways that regulate bone metabolism. However, it is not clear whether these genes can improve or maintain bone integrity, so validation studies are necessary to identify the functional role of these genes on BMD variation in patients with OA and OP, as well as analyze the effect that the suppression or activation of these genes has on the aforementioned phenotypes. In this performance, one of the main limitations is not to generalize the results obtained because of environmental factors among different populations such as diet, lifestyle, tobacco consumption, alcohol consumption, temperature, oxygen, humidity, cycle of light, presence of mutagens, which can influence changes in genetic expression profiles. Moreover, the effect of acupuncture could present variability between the operator, the technique, the type of stimulation, and the conditions in which therapy is developed, which has been recently demonstrated. However, through using of bioinformatics tools, computational theories, and mathematical models presented in this article, we propose to investigate and validate these models, as well as confirm our findings through clinical and experimental observations, combining molecular reductionism with quantitative holistic approaches to create a mathematical vision integrated OA/OP progression. In this sense, this work provides a list of new therapeutic targets that are sensitive to acupuncture treatment and that could be important study targets in monitoring this therapeutic form. Therefore, this *in-silico* analysis uses standardized methods to search for genes associated with different diseases and microarray analysis through the RMA method, which allows these results to be replicated.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Michell E. García-Espinosa: Writing–original draft, writing–review and editing and methodology; Paul Limias-Quezada: Writing–original draft, writing–review and editing and methodology; Alejandra I. Ortega-Meléndez: Writing–review and editing and methodology; Martha A. Ballinas-Verdugo: Investigation, writing–review and editing. Rosa E. López-Gómez: Data curation, writing–review and editing; Emma López-Espinosa: Writing–review and editing, Rogelio F. Jiménez-Ortega: Writing–original draft, conceptualization, methodology, formal analysis, investigation, data curation, writing – review and editing, supervision.

Ethical approval of studies and informed consent

Not applicable.

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Data availability

All relevant data are within the manuscript.

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