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Gene expression profile induced by ovariectomy in bone marrow of mice: A functional approach to identify new candidate genes associated to osteoporosis risk in women

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ABSTRACT

Osteoporosis is a multifactorial skeletal pathology with a main genetic component. To date, however, the majority of genes associated with this pathology remain unknown since genes cataloged to date only explain a part of the heritability of bone phenotypes. In the present study, we have used a genome-wide gene expression approach by means of microarrays to identify new candidate genes involved in the physiopathology of osteoporosis, using as a model the ovariectomized (OVX) mice by comparing global bone marrow gene expression of the OVX mice with those of SHAM operated mice. One hundred and eighty transcripts were found to be differentially expressed between groups. The analysis showed 23 significant regulatory networks, of which the top five canonical pathways included B-cell development, primary immunodeficiency signaling, PI3K signaling in B-cells, phospholipase C signaling, and FcgRIIB signaling in B-cells. Twelve differentially expressed genes were validated by MALDI-TOF mass spectrometry with good reproducibility. Finally, the association to bone phenotypes of SNPs in genes whose expression was increased (*IL7R* and *CD79A*) or decreased (*GPX3* and *IRAK3*) by OVX in mice was analyzed in a cohort of 706 postmenopausal women. We detected an association of a SNP in a gene involved in the detoxification of free radicals like glutathione peroxidase 3 (*GPX3*) with femoral neck BMD (rs8177447, $P = 0.043$) and two SNPs in the Ig-alpha protein of the B-cell antigen component gene (*CD79A*) with lumbar spine BMD (rs3810153 and rs1428922, $P = 0.016$ and $P = 0.001$, respectively). These results reinforce the role of antioxidant pathways and of B-cells in bone metabolism. Furthermore, it shows that a genome-wide gene expression approach in animal models is a useful method for detecting genes associated to BMD and osteoporosis risk in humans.

Introduction

The increased life expectancy that occurs not only in developed, but also in developing countries, has led to a higher incidence of degenerative disorders such as osteoporosis [1]. Osteoporosis is a common age-related systemic skeletal disease associated with low bone mineral density (BMD) and micro-architectural deterioration of bone tissue leading to an increased risk of bone-fragility fractures affecting both women and men [1]. Other features of this pathology are low bone mass, altered bone material composition and increased rates of bone remodeling. Among all these phenotypes, BMD is the best predictor of bone fracture, the major complication of osteoporosis that is responsible of the morbidity and mortality associated with this pathology [2].

Osteoporosis is a multifactorial disease and according to the WHO, the phenotype that defines it, BMD, is influenced by environmental, medical, genetic and also epigenetic factors [3]. Certainly, there is abundant evidence obtained from studies in twins, families and in epidemiological studies which reveal a significant genetic contribution to phenotypic variation in BMD. This is true as well in other phenotypes that are predictors of bone fracture, like skeletal geometry, bone turnover or the ultrasound properties of bone, with estimates of heritability ranging between 0.5 and 0.8 [4–6].

To find the underlying genes that regulate susceptibility to low bone mass and osteoporosis, different strategies have been used, including

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linkage studies, studies of candidate genes with *a priori* hypotheses, hypothesis-free genome-wide association studies (GWAS), and functional studies [4]. Linkage studies, given the multifactorial nature of the common form of osteoporosis, are not the best approach to the study of this pathology because of the low penetrance of the character [7]. Thus, like other complex diseases, most reports have been *a priori* hypothesis association studies with candidate genes. This approach has focused mainly on genes regulating bone metabolism and cytokines whose implication in bone metabolism has been well-known for some time. For example, *ESR1*, *COL1A1*, *VDR*, *TGFB1*, *IL-6*, *TNF-α*, *OPG*, and *LRP5* represent some of the approximately 150 candidate genes that have been investigated [4]. Recently, given the advances in high-throughput genotyping methods, GWAS have been performed in thousands of people comprising some 500,000–1,000,000 SNPs [8,9]. Although this type of approach has a number of known issues, *e.g.*, its extreme cost, limited statistical power or that it explains only a small percentage of phenotypic variance [4,10], these studies provide the scientific community with the ability to work without prior hypotheses. In general, they all point to a few biological pathways: Wnt/β-catenin signaling, estrogen endocrine, RANKL/RANK/OPG, bone ossification, mesenchymal-stem-cell differentiation, osteoclast differentiation and the TGF-signaling pathways [8,9,11–13]. Nevertheless, there about 30 BMD GWAS loci which lack prior molecular or biological evidence of involvement [12]. Finally, another approach consists of trying to define the mechanisms that underlie the association with phenotype performance, for example, animal models in which the gene has been mutated, over-expressed or deleted [10].

In this paper, we have analyzed the global gene expression through microarrays in bone marrow of OVX mice and compared it with that of SHAM operated control mice to identify genes showing differential expression in response to the estrogen deficiency induced by OVX. We decided to study bone marrow since it is in the microenvironment of this tissue where the main cells involved in bone homeostasis are located and because it represents a direct target of the effect of estrogen depletion. With this approach, we have tried to reproduce one of the major risk factors for osteoporosis in women, the estrogen deficiency that occurs at menopause or after a bilateral ovariectomy [14]. To our knowledge, we are the first to develop this translational research approach for studying the genetics of postmenopausal osteoporosis. The method consists of first identifying differentially expressed genes in response to OVX in an experimental animal model, and second, studying the association of some human genes orthologous to those differentially expressed in mice with BMD, in a cohort of women in order to establish whether they are susceptibility genes to postmenopausal osteoporosis.

Materials and methods

Mice and treatments

Fifteen-week-old, skeletally mature female C57BL/6 mice (Charles River Laboratories, Barcelona, Spain) were housed in an environmentally controlled laboratory upon arrival and acclimatized for 3 days. After this period, the animals were either dorsal ovariectomized (OVX, N = 23) or sham operated (SHAM, N = 14) under general anesthesia using 0.1 mg/kg Butorphanol (Torbugesic, Fort Dodge Laboratories, Girona, Spain) as a pre-anesthetic, 5% isoflurane to induce anesthesia and 1.5% isoflurane as maintenance (Veterinaria Esteve, Barcelona, Spain), and maintained as previously described [15]. One mouse from the SHAM group died as a consequence of the intervention. None of the other mice exhibited evidence of infectious disease, impaired growth, immunosuppression, or other side effects.

Four weeks after surgery the mice were sacrificed to obtain the right femur, having assessed the success of ovariectomy, as previously described [15]. One mouse of the OVX group was discarded because it did not show the expected uterine atrophy. The bone marrow cells were isolated from the femora of animals using centrifugation [16] then lysed immediately with 1 mL TRIzol (Invitrogen, Carlsbad, CA). All procedures for consideration of animal welfare were reviewed and approved by the ethical committee of our institution.

RNA isolation and GeneChip expression analysis

Total RNA from bone marrow cells was extracted using the TRIzol reagent and was purified using the PureLink Total RNA Purification System (Invitrogen) following the manufacturer's instructions. All RNA samples inside the purification column were treated with RNase-Free DNase for removal of contaminating DNA (Invitrogen). Purified total RNA was stored at −80 °C until used as a template for cDNA synthesis. RNA integrity was assayed by means of the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and RNA concentration was determined by measuring absorbance at 260 nm using a highly sensitive capillary spectrophotometer (GeneQuant, GE Healthcare Biosciences). Equal amounts of purified RNA extracted from 13 SHAM-operated mice and from 22 OVX mice were pooled in three (SHAM) and in five (OVX) pools, with each containing 4–5 different RNAs from individual mice. The generation of mRNA pools is a standard method to reduce the effect of biological replication [17]. Consequently, a total of 8 microarrays were developed and analyzed in the present study: three for SHAM mice (named C1–C3) and five for OVX mice (named O1–O5).

The synthesis of cDNA and cRNA, labeling, hybridization and scanning of the samples were performed as described by the GeneChip Expression Analysis Technical Manual (Affymetrix Ltd., UK), as previously described [18]. Twenty micrograms of fragmented biotinylated cRNA were used to prepare the hybridization cocktail and subsequently hybridized for 16 h at 45 °C for the Affymetrix GeneChip Mouse Expression Array 430 2.0, which contains of 45,101 probe sets, representing over 14,000 well-characterized genes. Arrays were washed, scanned and analyzed to obtain CEL files from pixel values on the DAT files, as previously described [18]. Global differences between different samples (CEL files) were measured by principal component analysis (PCA), linear discriminant analysis (LDA), and hierarchical clustering using the Partek Genomic Suite software (Agilent). For hierarchical clustering analysis, Pearson's dissimilarity was used to calculate row dissimilarity, and Ward's method was used for row clustering.

The analysis of significant changes in expression profiles of SHAM *vs.* OVX mice was done by GEPAS 4, a web-based tool (http://www.gepas.org) [19]. Briefly, for global background subtraction and cross array normalization, we used the Robust Multichip Average (RMA) method; for between array standardization methods, we used the quantiles tool, and for PM–MM adjustment we used the PM-only tool, and only PM values were taken into account [19]. The probe-set summary method was performed by using the median polish tool, which uses Tukey's median polish procedure to compute probe-set summaries. T-Rex was the tool used for analyzing differential gene expressions. It implements several modules to study gene expression under different experimental conditions. For each gene, this tool performs a t-test for the difference in mean expression between the two groups of arrays (SHAM and OVX), and T-statistics and *P*-values are reported. In the present work, significant genes from the comparison between SHAM and OVX mice were selected using the Benjamini–Hochberg method to control for a maximum false discovery rate (FDR) in the multivariate system. Only adjusted *P*-values <0.001 and a FDR used to discriminate false positives in the multivariate system <0.15 were considered as significantly different between groups.

The genes differentially expressed in response to OVX were visualized in biological pathways with the Ingenuity Pathways Analysis mapping software (IPA; Ingenuity Systems, Redwood City, CA; www.ingenuity.com). Data sets containing the Affymetrix probe set identifiers and T-statistics obtained from the t-test for the difference in mean expression between the two groups of arrays were uploaded to the application. In