Identification of TGFβ Signaling Pathway Genes Expression in Peripheral Blood and Bone Segments of Osteoporotic Postmenopausal Women

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Introduction

Osteoporosis is a skeletal disease with multifactorial pathogenesis, characterized by a combination of low bone mass and increase of bone fragility in the hip, wrist and vertebrae. The development of osteoporosis and the quantity and quality of bone are considerably influenced, apart from a large number of environmental factors, by genetic factors. TGFβ-BMP signaling pathway plays a significant role in bone development. Specifically, bone morphogenetic proteins (BMPs) and transcriptional regulators such as Runx2 are involved in bone regeneration and fracture healing. The genetic background of osteoporosis has been examined in numerous studies, but only limited data are available on gene expression profile of human osteoporotic patients.

The aim of the present study was to analyze the gene expression patterns of TGFβ-BMP-activated signal transduction and mRNA-levels of BMP2, RUNX2, genes in osteoporotic peripheral blood, bone samples and control postmenopausal women.

Material and Methods

Gene expression profile in bone samples was determined in 75 osteoporotic and 25 control postmenopausal Greek women. Menopause was defined as the absence of menstruation for at least 12 months. Bone mineral density (BMD) was measured at the total femur and at the lumbar spine (L2-L4) by dual X-ray absorptiometry (DXA). Bone markers, osteocalcin, calcitonin, insulin growth factor I (IGF-I), parathormon (PTH), leptin, were measured in the serum. Total RNA was isolated from peripheral blood using PAXgene Blood RNA kit (Qiagen) and from bone after osteoporotic fracture using TRIzol reagent (Invitrogen). The quality of the total RNA was determined by electrophoresis, while absorbance measurements were used to assess the purity of RNA.

In brief, cDNA was synthesized from 1-3 μg of total RNA using a cDNA Synthesis kit (Super Array Bioscience Corporation) and labeled with biotin-11-dUTP (PerkinElmer Inc.). Gene array expression analysis was performed by TGFβ-BMP Signaling pathway microarray (Super Array Bioscience Corporation). Each array was spotted with two blanks and 4088 cDNA targets as negative controls and four housekeeping genes: β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Heat shock protein 90kDa alpha (cytosolic), class B member 1 (HSP90AB1) and Beta-2-microglobulin (B2M). Each membrane was pre-hybridized with hybridization solution. cDNA probes were denatured and then hybridized with arrays spotted with cDNA fragments from 113 genes. The hybridized membrane was tagged with alkaline phosphatase-conjugated streptavidin, treated with CDK-Star and the expression was digitally captured using DNR MF-Chemibis Biomaging system after standardization of cameras’ parameters (DNR Bio-Imaging Systems Ltd). The relative abundance of a particular cDNA transcript was estimated by the subtraction of background spot intensity (average of three blanks) from the values recorded from each spot. Because the overall signal of different arrays may fluctuate substantially, the spot intensity was then normalized to the signal derived from housekeeping genes. The raw data were extracted and analyzed from the image using GEArray Expression Analysis Suite 2.0 analysis software (Super Array Inc.).

Expression of target genes (BMP2, RUNX2) was analyzed using a real-time quantification method (LightCycler) according to the manufacturer’s recommendations (Roche Diagnostics, Mannheim, Germany). Hybridization probes, used as detection format, consist of two different oligonucleotides. One probe is labeled at the 5’-end with LC-Red 640, the second probe is labeled at the 3’-end with fluorocsein (TIB MOLBIO, Berlin, Germany). Expression levels were determined as ratios between target genes and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. Our data were analyzed by Cluster analysis, K-means, ScatterPlot, Wilcoxon-Mann-Whitney and x-test.

Results

The median age of osteoporotic patients was 69 (range 56–75) years, T-score = -2.5 SD. The median age of the controls was 60 (range 45–75) years, T-score = -1.0 SD. IGF-I and osteocalcin were the only significantly different values among the osteoporotic and control group; however, IGF-I differentiated women with low BMD at greater level than the other studied bone markers (P<0.0001). A weak and none correlation was observed between leptin/BMD in patients (P=0.045) and controls (P=0.575), respectively, while no association was detected between calcitonin or PTH and BMD values at any group.

The TGFβ-BMP pathway over-expressed genes in osteoporotic peripheral blood, osteoporotic bone samples and control peripheral blood were compared and summarized in four categories: i) in osteoporotic, bone tissues vs peripheral blood: CDKN1A/ ENG/ SOX4/ SMAD1/ TGFBR3/ COLIA2/ FST/ ITGB5/ IGFBP3 (3-31 Fold Increase, FI); ii) in osteoporotic, peripheral blood vs bone tissues: PDGFB/ JUNB/ TIMP1/ JUN/ ITGB7/ FOS/ DLX2 (2-10 FI); iii) in peripheral blood, osteoporotic vs controls: STAT1/ TGFβ/ ITGB7/ ENG/ CDK1A/ FOS/ JUN/ MYC (2-4.6 FI); iv) in peripheral blood, osteoporotic vs osteocalcin: GDF11/ INHA/ LTP4/ TGFβ1/ RUNX1/ LEFTY2/ PDGFB/ HIPK2 (2-17 FI). Real-Time PCR analysis showed ratios of BMP2/GAPD (osteoporotic blood, median: 0.06; osteoporotic bone, median: 0.08; control, median: 0.05), and RUNX2/GAPD (osteoporotic blood, median: 1.14; osteoporotic bone, median: 3.81; control, median: 0.78) gene expression. RUNX2 and BMP2 expression levels in osteoporotic bone samples as well as in peripheral blood were significantly increased compared to controls.

Discussion

TGFβ-BMP signaling pathway plays a significant role in bone development; therefore, we decided to analyze gene expression alterations in peripheral blood and bone from osteoporotic and control postmenopausal women. Differences in gene expression profile of TGFβ-BMP signaling pathway were found between bone tissue/peripheral blood of osteoporotic and control women. Specifically, in osteoporotic samples CDKN1A (osteoblast expressed)/ FOS (SMAD target)/ TIMP1/Runx1, Runx2 target in hematopoietic cells) and JUNB (SMAD target/BMP responsive) were significantly expressed in blood as well as CDKN1A in bone, while in controls PDGFB (increases cell proliferation) and RUNX1 (TGFβ pathway regulator) (p<0.05).

The elevation of BMP2 (can induce osteoblast differentiation and bone formation) and Runx2 (specifically required for osteoblast differentiation) expression was further analyzed by Q-RT-PCR. Our results show significantly increased mRNA levels of the RUNX2 in osteoporotic bone segments and blood samples compared to controls. Although BMP2 expression levels were lower than RUNX2, they followed the same expression pattern among different sample types.

In conclusion, our data suggest the involvement of new candidate genes into the pathogenesis of osteoporosis. A combination of bone markers such as IGF-1 and osteocalcin, which differentiated the osteoporotic from the normal women and a further analysis of the above genes expression would be useful diagnostic marker for personalized drug therapy and fracture risk in osteoporosis.

Significance

Our study provides useful data on gene expression profile of human osteoporotic patients, which could be helpful in personalized therapy.