Gene expression changes in femoral head necrosis of human bone tissue

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Abstract. Osteonecrosis of the femoral head (ONFH) is the result of an interruption of the local circulation and the injury of vascular supply of bone. Multiple factors have been implicated in the development of the disease. However the mechanism of ischemia and necrosis in non-traumatic ONFH is not clear. The aim of our investigation was to identify genes that are differently expressed in ONFH vs. non-ONFH human bone and to describe the relationships between these genes using multivariate data analysis. Six bone tissue samples from ONFH male patients and 8 bone tissue samples from non-ONFH men were examined. The expression differences of selected 117 genes were analyzed by TaqMan probe-based quantitative real-time RT-PCR system. The significance test indicated marked differences in the expression of nine genes between ONFH and non-ONFH individuals. These altered genes code for collagen molecules, an extracellular matrix digesting metalloproteinase, a transcription factor, an adhesion molecule, and a growth factor. Canonical variates analysis demonstrated that ONFH and non-ONFH bone tissues can be distinguished by the multiple expression profile analysis of numerous genes controlled via canonical TGFB pathway as well as genes coding for extracellular matrix composing collagen type molecules. The markedly altered gene expression profile observed in the ONFH of human bone tissue may provide further insight into the pathogenetic process of osteonecrotic degeneration of bone.

Keywords: Osteonecrosis of femoral head, canonical variates analysis, human subjects, transcriptional profiling

1. Introduction

Primary femoral head osteonecrosis (ONFH) most commonly affects adults in the third and fourth decade of their life. More than one-third of the new cases compose the idiopathic form annually, and 5–12% of total hip arthroplasties each year are performed to treat this disease [1,2]. The condition is characterized by an insidious onset without specific clinical symptoms and signs. There continue to be significant gaps in our knowledge concerning the pathogenesis, natural history, early diagnosis, and optimal treatment of ONFH.

Osteonecrosis of femoral head is the result of an interruption of the circulation, which leads to a disparity between the oxygen need of the bone cell and the ability of the local circulation to supply that need [1]. The mechanism of ischemia and necrosis in non-traumatic ONFH is not clear. The majority of the studies focused on gene polymorphisms affecting the coagulation system. Positive correlation was reported of factor V, gene Leiden mutation with primary ONFH. Plasminogen-activating inhibitor-1 (PAI-1) gene 4G/4G genotype and 5,10-methylenetetrahydrofolate reductase (MTHFR) gene 677C/T heterozygote variant were over-presented in idiopathic ONFH patients. Furthermore, 4a/b variable number of tandem repeats (VNTR) polymorphism in intron 4 of nitric oxide synthase (eNOS) gene increased the risk of ONFH [3]. A comparative serum proteome analysis supports these genomic results and demonstrates that serum PAI-1 level is upregulated in
Fig. 1. X-ray (arrow) indicates the disease in the right femoral head at Ficat III stage when bone destruction appears.

ONFH [4]. Another ONFH-related candidate gene which may play a role in angiogenesis, is principally the vascular endothelial growth factor (VEGF) gene. Hong et al. [5] showed significant association of VEGF-FC polymorphisms with the increased susceptibility to ONFH.

Recent data suggest that synergistic actions of numerous genetic factors and exogenous agents (e.g. alcohol abuse, infections, disturbance of coagulation system or lipid metabolism, steroid usage) are also involved in the development of this multisystemic disease causing a primary form of ONFH. However, the genetic background as well as the cellular and molecular mechanism of the ONFH is still unclear.

The aim of the present study was to investigate the gene expression profile of those genes in human ONFH that have previously shown alterations in other metabolic bone diseases [6–10]. We assumed that performing a comprehensive gene-expression study we could identify important genes with significant role in the development of this condition.

2. Materials and methods

2.1. Human ONFH and non-ONFH control bone tissue samples

Gene expression pattern analysis in bone samples was determined in 14 unrelated, consecutive, Hungarian, Caucasian male patients. The collection of ONFH and non-ONFH control bone samples was achieved by our orthopaedist partners at Department of Traumatology and Orthopaedics, Csolnoky Ferenc Veszprém County Hospital. In case of ONFH patients, bone tissue samples were collected from the necrotic zone of femoral head during total hip arthroplasty before resection and were classified as grade III-IV, according to the Ficat Stages of Bone Necrosis of the Femoral Head. Preoperative radiograph images confirmed the diagnosis of ONFH in all cases (Fig. 1). Non-ONFH subjects participating in the study had undergone surgery due to traumatic hip fractures. Altogether, 6 ONFH and 8 control bone tissue samples were collected. Surgically removed bone samples were extensively washed in phosphate-buffered saline PBS for eliminating blood and marrow contamination and placed in liquid nitrogen. In all patients the serum alkaline phosphatase, TSH, PTH, osteocalcin, beta-crosslaps levels were measured. All subjects underwent physical examination and completed a detailed questionnaire on family and medical histories as well as lifestyle habits. BMD values at the total femur were measured using a Lunar Prodigy DXA (GE Medical Systems, Diegem, Belgium). Exclusion criteria were history of metabolic or endocrine diseases, any chronic illness, presence of cancer, previous glucocorticoid- or chemotherapy, alcohol abuse, any medication known to influence bone metabolism as well. The study was approved by the Regional Committee of Science and Research Ethics, Semmelweis University (6392-1/2004-1018EKU), and all patients gave written informed consent.
2.2. Direct polyA-RNA isolation

Human bone samples (approximately 500 mg) were cryo-grinded under liquid nitrogen using a freezer-mill 6750 (SPEX Certiprep Inc. NJ, USA). Five ml lysis / binding buffer from Dynabeads Oligo (dt)25 kit (Dynal Biotech ASA, Oslo, Norway) were added to the grinded bone tissue and it was centrifuged 9000 rpm for 15 minutes at room temperature. The separated median layer containing nucleic acid was added to 1 ml oligo-dt coated paramagnetic particles from Dynabeads Oligo (dt) coated paramagnetic particles from Dynabeads Oligo (dt)25 kit. mRNA was prepared using the buffer set according to the manufacturer’s protocol (Dynal Biotech ASA), it was eluted in 40 μl containing 1 μl cDNA, 10 μl TaqMan 2x Universal PCR Master Mix NoAmpErase UNG (Applied Biosystems, Foster City, CA, USA), 1 μl pre-designed and validated gene-specific TaqMan Gene Expression Assay 20x (Applied Biosystems) and 8 μl water. ABI Prism 7500 real-time PCR system (Applied Biosystems) was used to amplify the 117 selected genes from each sample in three parallel runs on a 96-well reaction plate with the following protocol: 10 minutes denaturation at 95°C, and 50 cycles of 15 seconds denaturation at 95°C, 1 minute annealing and extension at 60°C. Generally applied housekeeping genes (GAPDH and ACTB) were analyzed in every sample, and for further statistical evaluations the level of GAPDH was used as endogenous control for data normalization. Relative quantification studies were made from collected data (threshold cycle numbers, referred as Ct) with 7500 System SDS software 1.3 (Applied Biosystems). Relative quantity (RQ) of the gene specific mRNA was calculated from the averaged value of ΔCt-s (target gene Ct – endogenous control gene Ct) for each of the 117 gene transcripts in the two different study groups and were analyzed with 7500 System SDS software 1.3 from Applied Biosystem according to the manufacturer’s recommendations. Fold changes (RQ ONFH / RQ non-ONFH) were calculated from the RQ values in ONFH and control patients.

For univariate statistical analysis, we selected a non-parametric (i.e., distribution-free) method, the Student’s t-test. Results with a p value of 0.05 or less were considered statistically significant. Computations were performed using SPSS for Windows, release 13.0.1 (SPSS Inc. Chicago, IL).

2.3. Taqman probe based quantitative real-time PCR and the statistical comparison of the two groups of patients

Using quantitative real-time RT-PCR, we investigated candidate genes that might be involved in bone metabolism and osteogenesis with different expression activity. For comparing gene expression patterns in the bone tissue of ONFH and non-ONFH subjects, we selected 117 genes based on recent literature (Supplementary table), OMIM (Online Mendelian Inheritance in Man) database, as well as our data regarding genetic pathway analysis [6,9–11]. Twenty seven genes from the common TGF-beta/BMP pathway and Wingless (Wnt) cascade were selected for these studies; further 23 genes from extracellular matrix (ECM) components, 8 are involved in ECM degradation, 9 genes code growth factors, 5 genes code cell adhesion molecules, 7 genes code transcriptional factors and 12 of them are known to be controlled by estrogen and the 26 remaining genes were assorted by our previous published analyses [6,9–11].

Pre-designed and validated gene-specific TaqMan Gene Expression Assays from Applied Biosystem were used for quantitative real-time RT-PCR. Every set contained gene specific forward and reverse primers and fluorescence labeled probes. Probes span an exon junction and do not detect genomic DNA. The PCR reaction volume was 20 μl containing 1 μl cDNA, 10 μl TaqMan 2x Universal PCR Master Mix NoAmpErase UNG (Applied Biosystems, Foster City, CA, USA), 1 μl pre-designed and validated gene-specific TaqMan Gene Expression Assay 20x (Applied Biosystems) and 8 μl water. ABI Prism 7500 real-time PCR system (Applied Biosystems) was used to amplify the 117 selected genes from each sample in three parallel runs on a 96-well reaction plate with the following protocol: 10 minutes denaturation at 95°C, and 50 cycles of 15 seconds denaturation at 95°C, 1 minute annealing and extension at 60°C. Generally applied housekeeping genes (GAPDH and ACTB) were analyzed in every sample, and for further statistical evaluations the level of GAPDH was used as endogenous control for data normalization. Relative quantification studies were made from collected data (threshold cycle numbers, referred as Ct) with 7500 System SDS software 1.3 (Applied Biosystems). Relative quantity (RQ) of the gene specific mRNA was calculated from the averaged value of ΔCt-s (target gene Ct – endogenous control gene Ct) for each of the 117 gene transcripts in the two different study groups and were analyzed with 7500 System SDS software 1.3 from Applied Biosystem according to the manufacturer’s recommendations. Fold changes (RQ ONFH / RQ non-ONFH) were calculated from the RQ values in ONFH and control patients.

For univariate statistical analysis, we selected a non-parametric (i.e., distribution-free) method, the Student’s t-test. Results with a p value of 0.05 or less were considered statistically significant. Computations were performed using SPSS for Windows, release 13.0.1 (SPSS Inc. Chicago, IL).

2.4. Canonical variates analysis (CVA)

Univariate methods analyze the genes one by one, therefore many aspects in the data remain undetected. Multivariate methods may be called for in such situations. In this study, canonical variates analysis (CVA) was used to check whether the groups of ONFH and non-ONFH patients are separable in the multidimensional space spanned by the genetic variables, and if so,
3.1. Study population

The mean age of ONFH patients was 54.42 ± 2.57 years. The mean age of the control subjects was 60.88 ± 10.30 years. There was no significant difference in bone mineral density between the ONFH and control groups (Table 1).

3.2. Gene expression analysis in ONFH vs. control patients by Student’s t-test

We showed significant variation in transcription level of nine genes in our study. Summarized data for the fold changes (RQ ONFH / RQ Control) are presented in Table 2. COLIA2 (collagen type I alpha 2 chain), COL5A2 (collagen type V alpha 2 chain) COL10A1 (collagen type X alpha 1 chain), ITGA2 (integrin, alpha 2), KL (klotho), SP7 (osterix), TNC (tenascin C) genes were found to be over-expressed in patients with ONFH (Table 2). In addition, 2 genes namely MMP10 (matrix metalloproteinase 10) and VEGF (vascular endothelial growth factor) are showed significant down-regulated expression activity (Table 2).

Table 1
Clinical and biochemical characteristics of ONFH and non-ONFH subjects

| Mean ± SD | ONFH (n = 6) | Control (n = 8) | p value |
|-----------|--------------|----------------|---------|
| Age (year) | 54.42 ± 2.57 | 60.88 ± 10.30 | 0.13    |
| T-score total femur (SD) | −0.34 ± 0.71 | −0.86 ± 0.84 | 0.32    |
| Z-score total femur (SD) | −0.08 ± 0.79 | 0.12 ± 0.58 | 0.92    |
| BMD total femur (g/cm²) | 1.042 ± 0.09 | 0.979 ± 0.10 | 0.36    |
| Weight (kg) | 89.50 ± 23.41 | 77.50 ± 10.96 | 0.28    |
| Height (cm) | 180.20 ± 4.54 | 175.5 ± 7.58 | 0.26    |
| BMI (kg/m²) | 27.91 ± 8.26 | 25.08 ± 2.38 | 0.44    |
| Beta-CrossLaps (ng/l) | 286.37 ± 229.24 | 395.53 ± 195.73 | 0.44    |
| Osteocalcin (ng/ml) | 6.78 ± 8.15 | 4.40 ± 2.32 | 0.50    |
| Parathyroid hormone (ng/l) | 24.71 ± 6.00 | 26.35 ± 12.79 | 0.77    |
| TSH (mIU/l) | 0.81 ± 0.37 | 1.36 ± 0.60 | 0.06    |
| Alkaline phosphatase (IU/l) | 166 ± 28.52 | 157 ± 24.09 | 0.59    |

Probabilities (p values) in the right column refer to the results of the Student’s t-test for comparing the two samples.

Table 2
Summarized data for the fold changes of the significantly altered genes

| ABI Assay IDa | Gene symbolb | Gene nameb | Fold change | p valuec |
|---------------|--------------|------------|-------------|----------|
| Hs00164099_m1 | COLIA2       | collagen, type I, alpha 2 | 5.50 | 0.008    |
| Hs00169768_m1 | COL5A2       | collagen, type V, alpha 2 | 9.58 | 0.000    |
| Hs00166657_m1 | COL10A1      | collagen, type X, alpha 1 | 3.16 | 0.044    |
| Hs00158127_m1 | ITGA2        | integrin, alpha 2 (CD49B) | 3.77 | 0.014    |
| Hs00183100_m1 | KL           | klotho     | 4.00 | 0.050    |
| Hs00233648_m1 | MMP10        | matrix metalloproteinase 10 (stromelysin 2) | 0.008 | 0.050 |
| Hs00541729_m1 | SP7          | Sp7 transcription factor (osterix) | 28.55 | 0.000 |
| Hs00233648_m1 | TNC          | tenascin C (hexabrachion) | 4.89 | 0.004 |
| Hs00173626_m1 | VEGF         | vascular endothelial growth factor | 0.11 | 0.021 |

aApplied BioSystem TaqMan Gene Expression Assays Identification / Ordering Numbers.
bSymbols and names for human genes are used according to the standard “Gene Cards” (www.geneCards.org).
cFold changes (RQ ONFH / RQ Control). Changes of relative gene expression in ONFH patients compared to non-ONFH controls.
dp values of the Student’s t-test.

which gene subsets have the best discriminatory power. The results of CVA are canonical scores obtained from the canonical functions derived through eigenanalysis, which serve as coordinates of observations in the canonical space. Since the number of canonical axes is one less than the number of groups, in our case CVA produced only one variate. A partial limitation of CVA is that the number of variables cannot exceed the number of observations (patients). Computations were performed using the SYNTAX 2000 program package [12].

3. Results

3.1. Study population

The mean age of ONFH patients was 54.42 ± 2.57 years. The mean age of the control subjects was 60.88 ± 10.30 years. There was no significant difference in bone mineral density between the ONFH and control groups (Table 1).
Fig. 2. Canonical variates analysis of gene expression patterns of bone tissue in six ONFH (black bars) and eight non-ONFH, control (white bars) subjects. Symbols and names for human genes belonging to the nine subsets are used according to the standard “Gene Cards” (www.genecards.org). Gene symbols and correlations of genes with the single canonical variates (CV) are summarized in the table pertaining to each subset of genes.

3.3. Canonical variates analysis of ONFH samples

Nine CVA runs were carried out using different subsets of genes, each subset defined in a logical basis. The selection of four gene subsets, namely, WNT pathway (6 genes) [13,14] and BMP cascade (8 genes) [15,16], canonical TGFβ network (12 genes) [17] and MAPK/growth factor signaling (9 genes) was based on searching gene groups that are controlled via various signal transduction pathways. Two gene subsets contained two types of bone ECM forming components, collagens (14 genes) and non-collagen molecules (8 genes). The ONFH candidate genes group (6 genes) comprised of genes that has been shown to be in association with the pathogenesis of femoral head osteonecrosis [3,5,18,19]. We selected marker genes of bone cell metabolism and osteoblast/osteoclast differentiation and activation (9 genes) [20–23]. The remaining gene set included 7 genes that are involved in lipid metabolism [24–26].

Collagen type ECM-coding genes and genes belonging to canonical TGFβ pathway exhibited the best discriminatory power, with unambiguous separation of the ONFH and non-ONFH subjects (Fig. 2). Genes affected in the physiology of osteoblast and osteoclast cells, as well as genes coding for non-collagen ECM molecules also showed strong correlation with the single canonical variate, hence achieving clear separation of the two groups of the examined subjects. The subsets of ONFH candidate genes had lower discriminatory power (Fig. 2).

4. Discussion

Earlier, we applied a time and lab-consuming, but robust and highly reproducible quantitative real-time PCR method on several occasions for a comprehensive gene expression analysis of different metabolic bone diseases (e.g. postmenopausal osteoporosis, estrogenic hormone deficient human bone tissue and fibrous dysplasia). These genetic data from our laboratory have demonstrated significantly different expression patterns in various pathological conditions of bone tissue compared to the control physiological state [6–10]. In the present study, we have identified differences in gene transcription profiles of ONFH and non-ONFH bone tissue for the first time. We could sharply separate the physiologic and pathologic phenotypes based on genetic information. We have identified novel genes (SP7, COL1A2, COL5A2, COL10A1, ITGA2, MMP10, TNC, KL, VEGF) that show marked alterations in their expression levels, and most of those have not yet been related to ONFH.
Very clear segregation of subjects could be achieved by the gene subset of bone cell differentiation and activation (Fig. 2). This finding corroborates the results obtained by the Student’s t-test confirming the marked changes in the expression pattern of SP7. The transcription level of this essential osteoblast-specific transcription factor SP7 (osterix) was markedly higher in ONFH bone tissue. SP7 is necessary for pre-osteoblast differentiation into functional osteoblasts and acts downstream of another key factor RUNX2 in osteoblastogenesis [27,28]. RUNX2 can directly transactivate the SP7 promoter [29], and it is required for commitment of common mesenchymal progenitor cells to the osteoblast lineage [30]. Tingart et al. demonstrated markedly elevated RUNX2 mRNA levels in both the femoral head and neck region of patients with ONFH [31]. The stimulation of SP7 might lead to promoted osteoblast proliferation and activity in ONFH which is in accordance with the observation that osteoblast/osteocyte cell number is increased in ONFH bone samples [31]. We also measured increased RUNX2 expression activity in ONFH patients, however, the alteration was not significant. It should be noted that in the study of Tingart et al. [31], the control group was composed of osteoarthritic patients while they were healthy subjects in our case.

Canonical variates analysis revealed a relevant gene subset coding for collagen type extracellular matrix molecules (Fig. 2). Perfect sharp discrimination of ONFH and non-ONFH states was seen in case of these marker genes. In addition, we found that the expressions of COL1A2, the fibril-forming COL5A2 and the short chain COL10A1 were significantly increased in the ONFH bone tissue. These collagens organize the matrix texture. Collagen type V typically forms heterotrimeric collagen type X chains form hexagonal networks which bind calcium directly and play role in mineralization and matrix stability [33]. Collagen type 1 is the most widely expressed among the 29 genetically different collagen types in humans. One of the major genes determining bone strength is the collagen type 1α (COL1A1) gene. Type 1 collagen is a heterotrimer composed of two α1(I) chains and one α2(I) chain which are the major proteins of the bone. The α1(I) and α2(I) chains of collagen type 1 are encoded at the loci COL1A1 and COL1A2 respectively. Studies have described that during bone repair processes an over-expression of collagen genes can be detected, and newly formed healing bone can be characterized by the presence of COL1A expressed in active osteoblasts. We think the different expression of only one of the chains (α2) of type I and type V collagens might result in mechanical and structural changes in bone matrix. For example, the polymorphism affecting the binding site of the transcription factor Sp1 in a regulatory region of the COL1A1 gene was found to be associated with the risk of fracture in postmenopausal women. The higher expression level of COL1A1 gene is not the independent reason of increased fracture risk but the consequently increased production of alpha 1 procollagen relative to alpha 2 may result in reduced mechanical strength of bone [34].

At present, ONFH is considered to be the result of an interruption of the local circulation leading to bone destruction. We have observed changes in the expression of vascular endothelial growth factor (VEGF) genes in ONFH patients. VEGF an essential regulator for angiogenesis, plays key role in vascular cell migration and permeability. In particular, VEGF is important in bone formation process including blood vessel invasion and cartilage remodeling [5]. In addition, VEGF enhances blood flow in necrotic or avascular bone. Li et al. found that VEGF protein was highly expressed at the edematous area of the osteonecrotic femoral head adjacent to necrotic zone [18] as well as Radke et al. also showed rarely observed VEGF immunoreactivity in the necrotic zone [35].

MMP10, Tenascin C and ITGA2 are relevant extracellular matrix (ECM) composing elements and these genes control the function of bone organic matrix in different ways [36]. It is established that several principal bone cell-specific regulator factors – e.g. growth factor (BMP2, VEGF), transcription factor (RUNX2) – show altered action in ONFH, which might result in significant changes in bone homeostasis. The altered osteoblastic processes could partly mean shifted ECM formation, remodeling as well as impaired cell-ECM and cell-cell attachments in ONFH.

In conclusion, we have found significant differences in gene expression patterns of ONFH versus non-ONFH bone tissue. The separation of the two groups by CVA suggests the involvement of gene subsets that might be useful for a deeper understanding of the genetic aspects of this multifactorial disease. Our findings may provide further insight into the process of altered bone homeostasis in ONFH, and may promote wider application of an otherwise well-known statistical method (CVA) for the evaluation of batched genetic data.
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Supplemental material

Supplemental material can be found at: http://pentacorelab.hu/docview.aspx?r_id=3330373738&web_id=&mode=1.

Conflict of interest

All the authors hereby state that they do not possess financial interests and they have no conflicts of interest.

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