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Analysis of Gene Expression of Pseudoarthrosis Tissue in Congenital Short Femur, Initial Study and First Exprerience

Analýza genové exprese v tkáni pakloubu u vrozeně krátkého femuru, úvodní studie a první zkušenosti

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ABSTRACT

PURPOSE OF THE STUDY

Although the congenital short femur is morphologically well characterized, changes at the molecular level have not been described in the literature so far. The absence of such information, along with the unknown aetiology of the defect, was the motivation for analysing angiogenesis and osteogenesis in the pseudoarthrosis (false joint) tissue in PFFD patients compared to physiological bone. The authors expected differences in gene expression, particularly in the quantity of expressed genes.

MATERIAL AND METHODS

A piece of bone was removed during an elective surgery procedure, placed in an RNA stabilization reagent, which prevents RNA degradation, and deep frozen. Thereafter, RNA was isolated and the profile of transcription was analysed by biochip analysis (SuperArray Bioscience Corporation). In total, it is possible to detect 113 genes of osteogenesis and angiogenesis. From the end of 2005 until the end of 2008, samples of 7 patients with PFFD and 3 physiological bone samples were examined. Several analyses were repeated to confirm the results; in total 13 chips for osteogenesis and 11 chips for angiogenesis expression were used.

RESULTS

Differences in the quantity and representation of the genes were noted. Some genes were considered over-expressed in PFFD tissue compared with the control sample (e.g. the gene for calcitonin receptor, collagen XII, I alpha 2, collagen II, IX, FGFR2, fibronectin, integrin) and other genes under-expressed (e.g. the gene for annexin A5, collagen XVIII alpha1, collagen I alpha1, cathepsin K, FGFR1, FGFR3, IGF2, VEGFB).

CONCLUSIONS

The differences in gene expression confirmed the authors' hypothesis. So far, the results cannot be generalized; this is the first step for follow-up experiments to confirm the suggested information and to integrate it with clinical findings, such as the alternative blood supply of affected extremity in some patients.

Key words: proximal femoral focal deficiency, gene expression, microarray analysis, angiogenesis, osteogenesis.

The experiments were realised in cooperation with molecular biology laboratory of Institute of Pathology, Charles University and General University Hospital, Prague, Czech Republic, under leadership of Daniel Tvrdík, Ing.
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INTRODUCTION

The proximal femoral focal deficiency (PFFD) also called congenital short femur, is very rare but severe affection of the lower extremity. The morphological presentation represents the scale from completely absent femur to proportional femoral shortening, how presents Pappas´ classification (Fig. 1) (9). One of the most severe affection is congenital pseudoarthrosis of the proximal femur, and group of these patients became a target of our study. In 100% cases the femoral abbreviation is accompanied by at least one of other abnormality of the leg (dysplastic acetabulum, unstable knee, dysplastic patella, valgus knee with hypoplastic lateral condyle, abbreviation of the fibula, sometimes also tibia, ball in socket ankle joint, reduction of the foot rays). The clinical and radiographic appearance shows Fig. 2. Although several studies have been published on PFFD (2, 4, 6, 9), we have not found any study in international literature concerning the molecular analysis of imperfect bone tissue; only descriptions of histological pictures appear at the articles of Pappas (9) or Boden (1). Boden writes about "abnormal characteristics of the proximal femoral physis, which are based on the defect proliferation of

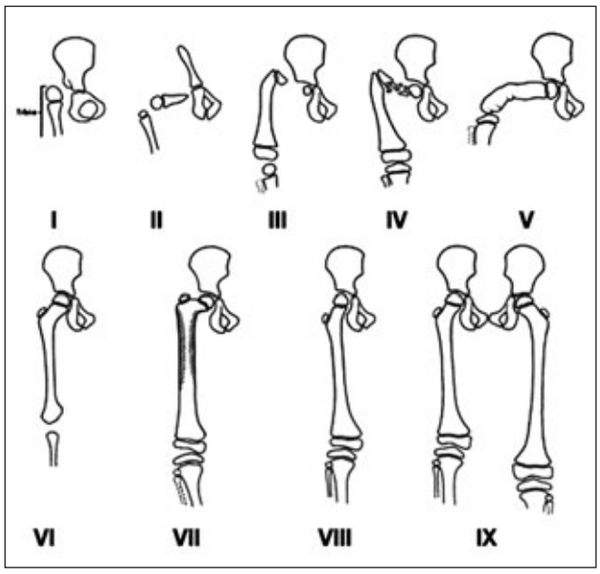

Fig. 1. Pappas' classification of proximal femoral focal deficiency (PFFD).

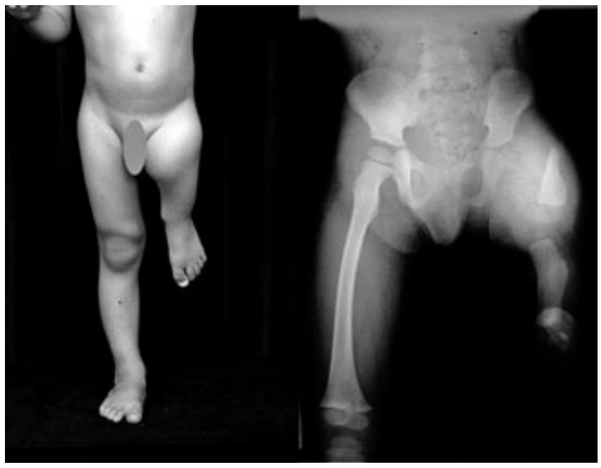

Fig. 2. Clinical and radiographical appearance of congenital short femur, type III according Pappas classification (fibrous pseudoarthrosis of the femur and severe shortening). 5 years old patient with shortening of left lower extremity 24 cm, on radiogram discontinuity of the femur, fibular aplasia on the left side.

chondrocytes". As a sequel, he mentions bone matrix mineralisation delay, disruption of vascular invasion and endochondral growth. On the basis of Boden's work, speculation can be made regarding the background of bone abnormalities resulting from changes in bone development at the gene level, particularly in the group of genes applicable to proliferation and differentiation (e.g. BMP – bone morphogenic protein, VEGF – vascular endothelial growth factors, FGF – fibroblast growth factor), in the group of genes ensuring the bone mineral balance (e.g. genes for calcitonin receptor, annexin A5, metalloproteinases) and also in genes for angiogenetic factors. However, osteogenesis is a highly complex process directed by both the intracellular and extracellular environment. The regulation of these processes presents numerous growth factors, signal molecules and receptors, whose function depends on its gene expression. If the production of any factor fails completely or the factor is made imperfect, then errors in osteogenesis can occur. It is clear, that osteogenesis is not an isolated process and appears to be influenced strongly by angiogenetic factors. For example, it has been proven that FGF-2 induces the production of VEGF in osteoblasts, as does BMP-2 (12,13).

The above facts led us to the idea of undertaking the study, which aim is a gene expression analysis of osteogenic and angiogenetic factors in pseudoarthrosis (false joint) tissue in patients with PFFD. As we premised in preliminary results of the project in 2009 (5), we expected to find differences in gene presentation in pseudoarthrosis tissue compared to normal physiological bone tissue, both in quantity and types of genes (representatives of various osteogenesis and angiogenesis phases). It could clarify the difficulties and complications during bone procedures, especially the limb lengthening and bone healing, in the patients with congenital limb deficiency (for example congenital short femur, congenital tibial pseudoarthrosis or fibular hemimelia).

MATERIAL AND METHODS

Our group of patients counts 81 patients with congenital short femur. This unique group content the whole scale according Pappas classification, with the most patients in VII-IX class. In preference we chose for molecular analysis the patients with the severe defect of the femur, called also congenital pseudoarthrosis of the femur (Pappas group III and IV). The appearance of the severe classes in new-borns is very rare (1–2:100000), so the number of patients for analysis is rather small.

The pieces of physiological and pathological bone tissue were obtained during elective surgery, with the informed agreement of the patients (or their parents). The pseudoarthrosis tissue was removed when the resection of pseudoarthrosis and the fusion of proximal femur were performed in PFFD patients. The physiological bone was obtained after resections in foot surgery (tarsal coalition resection), or during pelvic graft extraction in patients for whom a diagnosis other than PFFD was the reason for surgery.

Immediately after extraction in the operating theatre, bone pieces of 5x5mm size were placed into preservation fluid (RNA Safer, SuperArray Bioscience Corporation) and were frozen to 80°C. After unfreezing, it was necessary to crush the bone mechanically to allow extraction fluids to enter. For this purpose, we first used a sample splitter, and later changed to grinding in mortar after freezing the samples with fluid nitrogen. The isolation of RNA was performed using the RNeasy Mini Kit (Qiagen), according to the company recommendations. The kit combines lysis of the sample by guanidine isothiocyanide and linkage of RNA to silicate membrane,

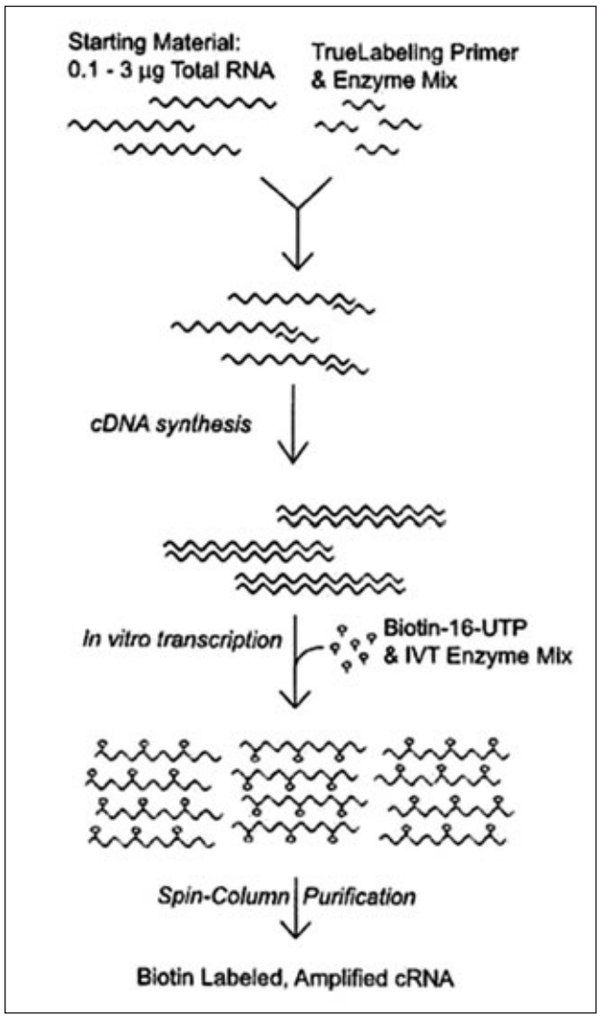

Fig. 3. Amplification and RNA labelling by biotin. Isolated RNA from specimen are mixed with enzyme and primers, inserted into the thermocycler, where, after several hours, cDNA synthesis by reverse transcription is run. Biotin with the enzymes is added to the obtained material and during the following several hours of reaction, DNA undergoes transcription to RNA, RNA amplification and biotin labelling. Biotin is incorporated into the structure of RNA by binding with uridine (timidine analogue) and pairing with adenine (basic building elements of DNA and RNA).

from which clear RNA is eluted after washing. To receive a larger amount of RNA, we attempted to use enzymatic lysis of the samples before RNA isolation (enzyme mixture: elastase, trypsin, collagenase II, collagenase VII, collagenase IX). However, as this did not result in an increase in RNA quantity, we did not continue further with enzymatic lysis.

Further RNA manipulation was applied according to the guidelines from SuperArray Bioscience Corporation, whose biochip technology we selected for our experiments. Essentially, it concerns the simultaneous analysis of RNA expression based on immobilisation of DNA probes (DNA molecules specified for catching the other molecules) on the membrane, where DNA probes are

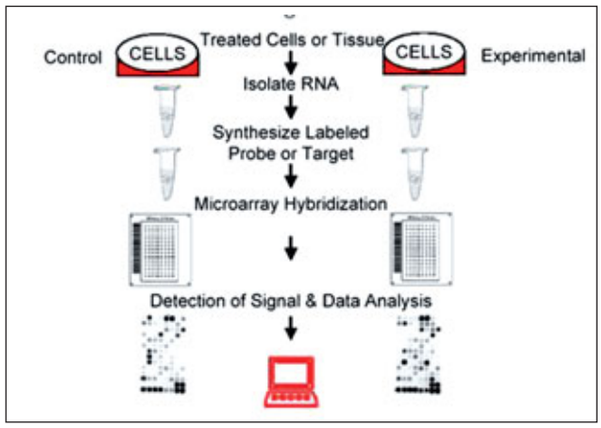

Fig. 4. Experiment procedure. Simultaneous analysis of the specimen and control is performed. After the preparation of RNA following the procedure in Fig. 1, hybridization is undertaken. The RNA specimen labelled by biotin with the mixture of reagents is added to a biochip membrane, in which sequences of the osteogenic and angiogenic genes are anchored. If the complementary sequences are present, the hybridization arrives. Chemiluminescent agent is added to the binding sequences and the signal can be detected on x-ray film. The spectrum of visible signals, which represents the expressed genes, is analysed by software.

regularly organized into rows and columns. In the next step of the analysis, DNA probes are exposed to the reaction of the corresponding molecules of the samples. Thus the binding complexes arise, which, after washing, are detected by a chemiluminescent reading system. In one reaction, the system allows the detection of up to 113 genes of angiogenesis or 113 genes of osteogenesis.

RNA isolation was followed by cRNA synthesis (complementary RNA), biotin RNA labelling and amplification (Fig. 3). After purification of the amplified and labelled RNA sample, it was necessary to prepare biochip membranes for hybridization (pre-hybridization). Then samples of labelled RNA were added into the hybridization tubes. During hybridization, complementary parts of RNA were bound, expressed in the tissue. This part of the experiment took place over night in a hybridization oven at 60°C. After washing, the membranes were incubated with the fluid of streptavidin conjugated with alkaline phosphatase, and the detection of the chemiluminescent radiation generated by the reaction of CDP-star (chemiluminescent colour) with the conjugate alkaline phosphatase-streptavidin-biotin-cRNA-biochip membrane. Radiation was recorded on X-ray film. The scan of the x-ray film was evaluated by GEArray Expression Analysis Suite (SuperArray) software. The method also allows the semi-quantitative analysis of expressed genes depending on the radiation intensity. The experiment procedure is shown in Figure 4.

At the beginning of the project, we calculated the frequency of the severe variants of PFFD with developed pseudoarthrosis, i.e. about 1–2 cases for 100,000 newborns (2, 4, 6). During the project (2008–2012), 7 sam-

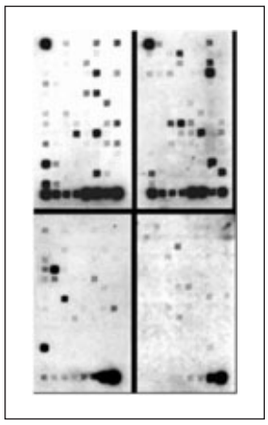

Fig. 5. The result of biochip analysis in the control sample of physiological bone (upper pair of membranes) and in a patient with PFFD (bottom pair). In the left column are the results of hybridization of osteogenic factors; on the right side angiogenic factors. On the bottom membranes fewer signals are visible, representing fewer expressed genes in the patient with PFFD and pseudoarthrosis. This confirms our hypothesis about differences in gene expression.

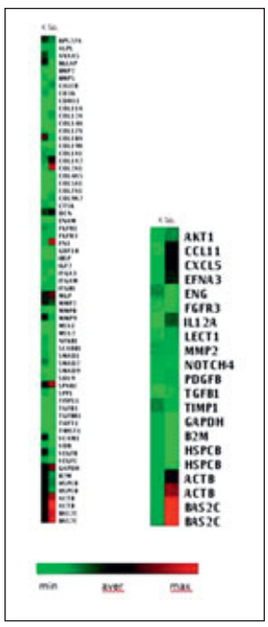

Fig. 6. Semi-quantitative analysis of the spectrum of expressed genes. In two pairs of columns, the control sample is always on the left side; the sample of the PFFD patient is on the right. The left pair represents the spectrum of osteogenic expression, where over-expressed genes were delegates of "unmatured" bone with a gene for collagen II, collagen XII, collagen IX, calcitonin receptor, FGFR2, fibronectin and integrin. Under-expressed genes related to the stimulation of angiogenesis (VEGFB, VEGFC, FGFR1, FGFR3), genes for collagen I subunit alfa 1, and from the next e.g. cathepsin K, vitamin D receptor, annexin A5. The colour scale shows the semi-quantitative amount of genes according to the adjacent legend.

ples of PFFD patients and 3 samples of controls were analysed, some of which were analysed repeatedly. In total, 13 biochips for osteogenesis and 11 chips for angiogenesis were used. Most of the pathologic bone pieces came from patients with Pappas III type deformity (4 patients), with individual samples from patients with types II, VI and VII. The structure of the group is shown in the table.

RESULTS

The expressed gene spectre was highly heterogeneous on membranes (biochips). However, our initial hypothesis that both the amount and representation of the genes was different in the physiological tissue compared to imperfect bone tissue of a PFFD patient was confirmed. Generally, fewer genes were expressed in pathologic bone tissue than in the physiologic sample. In the most representative pair of biochips, the percentage of expressed osteogenic genes was 35% in the physiological bone piece compared to 26% in the pathologic bone sample; angiogenetic genes were expressed in 33% of the panel in physiologic bone and in 16% in pathologic tissue (Fig. 5).

The software analysis of osteogenic genes indicated over-expression of several genes in pathologic bone compared to physiologic bone, e.g. calcitonin receptor gene, collagen XII gene, collagen I subunit alfa 2 gene, collagen II, collagen IX, FGFR2, fibronectin and integrin genes. Conversely, several genes were under-expressed: annexin A5 gene, collagen XVII subunit alfa 1, collagen I subunit alfa 1, cathepsin K gene, FGFR1, FGFR3, IGF2, VEGFB, VEGFC.

In the angiogenetic group of genes, we noted an especially marked difference in quantity, with fewer genes expressed in pathologic tissue. After software biochip analysis, we detected a lack of certain angiogenetic genes in the tissue of PFFD patients, e.g. VEGFB, VEGFC (vascular endothelial growth factor B and C), ECGF 1 (endothelial cell factor 1), genes encoding other growth factors and receptors (e.g. ANPEP, LAMA5, PDGFA), proteinases and inhibitors (PECAM 1, PF4), transcription factors (PTEN, PTGS1) or adhesive molecules (CDH5, COL18A1, LAMA5) (Fig. 6). Summary of genes over or under expressed in patients with PFFD in comparison to controls shows Table 1.

Table 1. The overview of expressed osteogenic and angiogenic genes in patients with PFFD in comparison to control samples. In the first column (osteo+) are the genes with over-expression in PFFD patients, the second column (osteo -) presents under--expressed genes. In the column signed "angio +" are angiogenic genes over-expressed in PFFD patients, in the last column are noted genes completely absent in gene expression profile in PFFD patients

osteo +	osteo -	angio +	angio A
CALCR	ANXA5	CCL11	VEGFB
COL12A1	BGLAP	CXCL5	VEGFC
COL1A2	COL18A1	EFNA3	ECGF1
COL2A1	COL1A1	IL12A	ANPEP
COL9A2	CTSK	PDGFB	LAMA5
FGFR2	FGFR1		PDGFA
FN1	FGFR3		PECAM1
ITGAM	IGF2		PF4
	ITGA3		PTEN
	ITGB1		PTGS1
	MMP2		CDH5
	MMP8		COL18A1
	MMP9		
	VEGFB		

Unfortunately, in the evaluated samples we were not able to detect stable differences in the expression of osteogenic and angiogenetic genes, which could be confirmed for example by immunohistochemistry. This could be due to the conglomerate of cell-specific types

in pseudoarthrosis tissue, where chondrocytes and fibrous cells could predominate the osteocytes.

During the experiments, we encountered several complications, the most significant of which was the very low quantity of RNA acquired from tissue isolation. Occasionally, even if the RNA samples were amplified and concentrated, the amount of RNA in reaction was on the lower border of the recommended limit for the procedure. In one case, we did not have sufficient material to analyse both groups of genes. We attempted to increase the quantity of RNA using enzymatic lysis of the mechanically prepared sample before the proper isolation of RNA, but as the yield of RNA was not significantly higher, we abandoned this procedure. Another approach to increase the yield of RNA was establishing osteoblast cell culture. This method not only provided more RNA quantity, but also acquired cell-specific RNA of various types of cells, which are presented in bone tissue (osteoblasts, chondrocytes, fibrous cells). In 2008, we established the cell cultures of physiologic osteocytes to introduce and adopt the method. However, the method of cell cultures did not become a routine part of the experimental procedure due to early termination of financial support. This method remains target for future experiments.

DISCUSSION

With regard to the gene expression in pathologic bone tissue, specifically pseudoarthrosis in congenital short femur, we have not found any link in the literature to which we could connect or challenge in light of our findings. Consequently, it is difficult to evaluate and comment on the findings of our study on differences in both osteogenesis and angiogenesis in patients with PFFD compared to control samples of physiological bone. As we cannot continue our experiments, we restrict our conclusions to reflections on the possible relationships of our results with clinical consequences. It is necessary to confirm our hypothesis in future experiments (f.e. immunohistochemistry, proteomic analysis, cell specific RNA analysis).

Consistent with our primary expectation, in pseudoarthrosis tissue fewer genes were expressed than in the physiological control sample. The question arises as to whether it represents a real reduction of the gene expression of angiogenetic and osteogenic factors, or whether the reason is a smaller number of expressed genes in a mixture of other cell types in pseudoarthrosis tissue (chondrocytes, fibrotic cells, myoblasts). This assumption could be confirmed by examining cell-specific RNA obtained from the cell cultures of separate cell types.

Overall, we noted a smaller amount of expressed angiogenetic genes. We can find a possible explanation for this phenomenon in the anatomic anomaly in vessels supplying the affected extremity in PFFD patients. In two patients, we confirmed this situation by CT angiogram scans (7, 8).

The majority of patients with proximal femoral focal deficiency show distinct vascular changes in the involved extremity, including reduction of the diameter and length of the arteries in contrast to the unaffected, contralateral extremity. The diameter of the femoral artery was reduced more in the patients with more severe forms of proximal femoral focal deficiency. On the other hand, there were no changes in the vascular pattern that could be described as specific for a particular type of proximal femoral focal deficiency (acc. Pappas classification). The changes in the topographical anatomy of the vessels supplying the extremity in proximal femoral focal deficiency were found in only two patients. In these patients, the inferior gluteal artery continued as the artery to the sciatic nerve and then formed the popliteal artery as the main supply of the distal end of extremity. The difference in these two patients could be attributed to the persistence of an embryological pattern of the vascular supply of the lower extremity. However, the question as to *why* remains unanswered. What is first: the bone or vascular defect? Is it a local or systemic reason? Is it mechanical, genetic or another reason?

The primary vascular defect of the proximal femur in the location of pseudoarthrosis could explain why important angiogenetic factors are not "delivered" into the developing bone, which stimulate the production of osteogenic factors. On the other hand, some of the osteogenic factors are necessary for the induction of angiogenetic factors.

It is known from stem cell studies that VEGF (vascular endothelial growth factor) plays an important role in bone formation induced by BMP-4 (10, 11). VEGF participates in bone growth in the endochondral ossification process. The VEGF inhibition leads to the depression of trabecular bone formation in the growth plate secondarily after the decrease of blood vessel invasion and deterioration of cartilage resorption (10).

The connection between angiogenesis and osteogenesis has been carefully analysed at Stanford University (12). One of the known connections between angiogenic and osteogenic factors is the induction of osteoblasts by FGF-2 (fibroblast growth factor), which then produce both VEGF and BMP-2. Farhadi also present the same conclusion. In their study with human stem cells in vitro they observed up-regulation of BMP-2, TGF beta 1 (transforming growth factor) and VEGF expression caused by FGF-2 (3).

In an interesting study published by the Stem Cell Institute in Minnesota (11), the authors investigated the differentiation of human mesodermal progenitor cells in vitro. After 7 days of cultivation it was possible to detect by immunohistochemistry methods osteocalcin and type I of collagen on differentiated osteoblasts, but not collagen type II. However, in a culture of differentiated chondrocytes, immunohistochemistry showed the presence of collagen type II and aggrecan, but type I of collagen and osteocalcin were not detected (present).

In line with the above result, in our specimens we detected a lower expression of type I collagen in the pseudoarthrosis tissue compared with a physiological bone piece, while the expression of collagen II was higher. This shows the greater representation of chon-

drocytes in pseudoarthrosis tissue, and consequently the reduction of mechanical resistivity. Collagen I alfa 1 down-regulation in expression is also noted by Steinbrech in their experiment during membranous healing of the jaw after osteotomy in rats (13).

CONCLUSIONS

We are aware, that our results at this stage unfortunately do not allow us to conclude the issue of osteogenesis and angiogenesis of the samples analysed. We are in the initial phase of a long and complicated process to interpret and present our findings with certainty and relate them to clinical consequences. We believe that further experiments could contribute to understanding not only the aetiology of congenital short femur, but also bone development in general; the information gained could be helpful in tissue engineering development. Moreover, it is necessary provide the bigger amount of analyses for statistical evaluation of the results.

We intend to perform cell microdissection from histologic specimens and then isolate the cell-specific RNA. Subsequently, RealTime PCR analysis can detect the expressed genes and their amount. The next possible method to acquire cell-specific RNA from the present individual cell types is establishing cell culture.

Our aim is to confirm the hypothesis that osteoblasts of congenital damaged (false founded) tissue shows a different gene expression than physiological healthy bone, and to discover key genes with markedly different expression. We assume that false bone development is established by the disruption of gene regulation supported by local factors. A further experimental plan is to study whole-genomic abnormities in the DNA or RNA of parents and children with PFFD, and possibly also comparing the DNA of healthy individuals with the DNA of PFFD children (comparative genomic hybridisation). This method allows a comparative analysis of the chromosomal changes in the whole genome and can detect amplification or loosening of genetic material according to changes in fluorescent signal intensity. The results could direct us to the specific part of the genome for further analyses and studies.

The results of our study represent an initial step and contribute to further research in the field of development and differentiation of tissues. Deeper understanding of the bone healing mechanism could also support healing in clinical practice.

Conflict of interest statement: none declared.

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