Mutation Analysis of the COL1A1 Gene in Czech Patients Affected by Osteogenesis Imperfecta, Type I-IV

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Abstract

Background: Osteogenesis imperfecta is a worldwide widespread disorder of connective tissue characterized by extensive clinical heterogeneity. The main clinical feature is increased bone fragility due to defective collagen type I production which is encoded by two genes – COL1A1 and COL1A2. Based on clinical, radiological and genetic features there is described 11 forms of the disease. Only the first four types result from the collagen type I mutations. Severity of the disorder ranges from mild to lethal forms.
Objectives and Methods: The aim of this study is the molecular-genetic analysis of COL1A1 gene of 25 Czech patients suffering from the disease named osteogenesis imperfecta, specifically type I-IV, and comparison of clinical pictures of individuals with the same identified mutations.
Results: COL1A1 gene mutations were identified in three of twenty-five Czech OI patients. These individuals come from unrelated families and are affected by osteogenesis imperfecta type IA, III and IVB.
Conclusion: Further molecular-genetic analyses of other patients and their relatives are important for detection of the biggest mutational spectrum necessary for determination of possible genotype phenotype relationship of affected individuals and for comparison the Czech population with others countries.

Keywords

Osteogenesis imperfecta, collagen type I, COL1A1, COL1A2, MLBR, mutations

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1 Introduction

Osteogenesis imperfecta (OI) is a heritable disorder of connective tissue. Hallmark feature of the disease is increased bone fragility with increased risk of fractures. Other associated signs are subnormal to low stature, joint hypermobility, skin hyperlaxity, blue sclera, hearing loss and dentinogenesis imperfecta. Some patients suffer from pulmonary or vascular defects.

The first classifications created in 1979 by David Sillence included four clinical different OI types. Current classification distinguishes eleven forms on the basis of clinical, radiological and genetic signs. First four types result from collagen type I genes (Collagen, type I, alpha-1 (COL1A1) and Collagen, type I, alpha-2 (COL1A2)) mutations.

Origin of remaining types are mutations of the gene Serpin peptidase inhibitor, clade F, member 1 (SerpinF1) (OI type VI), genes of the 3-prolyl hydroxylation complex - Cartilage associated protein (CRTAP), Leucine- and proline-enriched proteoglycan 1 (LEPRE1) and Peptidyl-prolyl isomerase 1 (Cyclophylin B) (PPIB) (OI types VII, VIII and IX), and defects of chaperones Serpin peptidase inhibitor, clade H, member 1 (SerpinH1) and FK506-binding protein 10 (FKBP10) (OI types X and XI). Etiology of the fifth OI type is currently unknown [5, 18].

1.1 Collagenous Forms of Osteogenesis Imperfecta

OI type I is the mildest form of OI inherited by autosomal dominant manner. The risk of fractures is increased in childhood, after woman's menopause and after 60th year of life in men. Individuals are normal stature, have mild or no deformities, may have blue sclera or suffer from hearing loss. Some of them have dentinogenesis imperfecta (DI). This feature distinguishes type IA (absent DI) and type

IB (presence DI) of OI. Presence of all clinical signs is very variable [9, 17].

OI type II is a perinatal lethal form. Stillborn is common, perinatal mortality occurs in 80% of cases in the first week of the life. First fractures occur in the uterus, patients have severe deformed bones, triangular face and blue or grey sclera. There are three subtypes of this form – types IIA, IIB and IIC, differentiated according to radiographic features such as deformity of ribs and long bones and cephalometric features (macrocephaly, microcephaly), type of heredity and mutated gene (autosomal dominant types IIA and IIC result from mutations of COL1A1 and COL1A2 genes, the autosomal recessive type IIB is caused by mutation of CRTAP gene) [2].

The third type of the disease is moderately deforming form of OI with autosomal dominant or recessive inheritance. Patients achieve subnormal body height. They have short extremities, severe deformities of bones, hypermobile joints, triangular face, dark blue sclera (turn white in adulthood) and DI. Typical radiological features are wormian bones of skull and popcorn calcification of epiphyses and metaphyses of long bones. Severe scoliosis, thin diaphyses of long bones and high frequency of fractures during normal daily activities are the main reason for using of the wheelchair [13].

OI type IV is the most heterogeneous type of this disorder with autosomal dominant inheritance. Growth retardation is moderate to severe, affected individuals have bowing bones, popcorn-like structure of epiphyses is less common than in the OI type III. First fractures may occur at birth, sclera is white, blue or grey and some patients suffer from otosclerosis. Typical clinical feature is basilar impression. Based on presence of DI we distinguish the types IVA (absent DI) and IVB (presence DI) [7].

1.2 Non-Collagenous Forms of Osteogenesis Imperfecta

OI type V is the autosomal dominant osteogenesis imperfecta type with unknown genetic origin. It is moderate deforming form which presents with hypertrophic callus formation in areas of fractures and with interosseous ossification of the forearm bones [5, 12].

The sixth type of the disease is inherited by autosomal recessive manner. It is a progressive deforming disorder characterized by presence of bone lamellae like fish scale, osteopenia, long bone deformities and bulbous metaphyses [12, 13]. It is caused by SerpinF1 gene mutations [5].

Type VII OI is an autosomal recessive OI form with severe to lethal clinical manifestation. Main signs of this type are rhizomelic shortening of humerus and femur and exophtalmos. Frequency of fractures decreases throughout adulthood. It results from CRTAP gene mutations [16].

Next autosomal recessive form is OI type VIII. Phenotype of affected individuals is various from severe to lethal. The typical clinical feature is rhizomelic shortening of extremities. Other radiological features are bulbous epiphyses, osteoporosis and shortened long bones. Causative gene of this OI type is LEPRE1 [4, 5].

Osteogenesis imperfecta type IX, the moderate to lethal form, resembles with its clinical picture the III and the IV type of the disease. Familial transmission of the disorder is autosomal recessive. This type results from defects in a PPIB [9].

A severe to lethal OI type X, the autosomal recessive form of the disease, results from SerpinH1 gene mutations. Phenotype of individuals is presented by rhizomelic shortening of extremities like in types VII and VIII [5].

The last type of OI is the type XI. It is a progressive deforming form inherited in autosomal recessive manner and caused by defects of a FKBP10 gene. Typical clinical features are bone lamellae like fish scale as well as in the sixth type of the disorder [5].

1.3 Molecular-Genetic Origin of Osteogenesis Imperfecta

80-90% of OI cases are caused by mutations in one of two collagen type I genes - COL1A1 and COL1A2. The molecule of the protein is composed of two alpha1 chains encoded by the COL1A1 gene localized on chromosome 17 and one alpha2 chain encoded by the COL1A2 gene situated on chromosome 7. The unfolded chains undergo several modifications (4-prolyl hydroxylation, 3-prolyl hydroxylation, lysine hydroxylation, glycosylation) increasing stability of the molecule. Such modified alpha chains fold in the direction from the C-terminus to the N-terminus in a heterotrimer terminated by C- and N-propeptides (this is the reason of more severe disability in individuals whose collagen is mutated in the C-region of the molecule) [1, 9].

The most important amino acid in the alpha chain is glycine (Gly) that produces inter-chains links. It is contained in every third position in 338 repetitive Gly-X-Y sequences and is required for correct alpha chains folding into the triple helix formation. About 75%-80% of structural defects of collagen type I result from substitution mutations of another amino acid instead of glycine [10]. 36% of COL1A1 glycine substitutions are lethal while in COL1A2 gene 19% of mutations of this amino acid have lethal outcome [5].

The other crucial areas of the alpha chains are the transcription factors binding sites - activating proteins (so-called enhancers and silencers) binding sites whose binding to the alpha chain activates or inhibits transcription [6], CpG rich areas can undergo methylation resulting

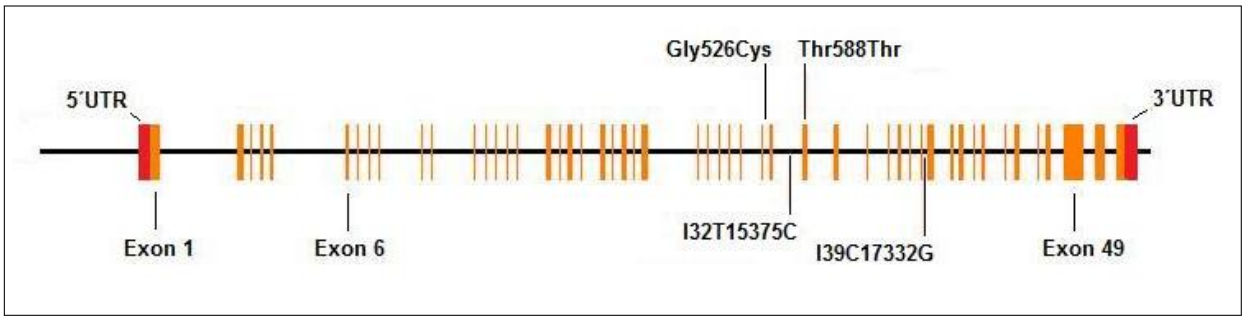

Figure 1: Overview of identified mutations/polymorphisms in the gene COL1A1.

in moderate phenotype if it occurs in promoter, exon 1 or intron 1 and in severe clinical picture if this occurs in the coding sequence of COL1A1 and COL1A2 genes. This process can happen in 26 of the 338 glycine codons of the alpha chains [8].

Finally, there are three Multi Ligand Binding Regions (MLBR1-3) producing intermolecular linkages with other molecules of the connective tissue, for example integrins, Cartilage Oligomeric Matrix Protein (COMP), SerpinH1 and other. These interactions increase strength and flexibility of bones. Mutations of MLBR2 and MLBR 3 result in most cases in lethal osteogenesis imperfecta [5, 15].

1.4 Treatment of Individuals Affected by Osteogenesis Imperfecta

Treatment of patients with osteogenesis imperfecta is different and individual based on concrete clinical, biochemical and radiological picture. Medical treatment includes calcium, vitamin D and bisphosphonates therapy. Bisphosphonates are the most commonly used medicaments for moderate and severe forms of OI. Their specific function is inhibition of osteoclasts on the surface of bones leading in increase of bone mineral density and decrease of risk of fractures [3].

Orthotic treatment is introduced for patients with scoliosis and mild deformities of extremities, while severe deformities and fractures with significant displacement are treated surgical using osteotomy and fixation with intramedullary rods, nails, pins etc. Severe scoliosis is surgically resolved by fixation with Harrington rods, however, this procedure greatly reduces subsequent range of motion of the spine.

At present, methods called cell and gene therapy are being developed. The aim of these methods is replacement of defective osteoblasts with subsequent increasing of bone mineral density (cell therapy) and deactivation of the mutated gene resulting in decreasing of OI severity (gene therapy) [9, 11].

2 Materials and Methods

We analyzed in this research gDNA samples obtained from whole blood of the 25 Czech patients (four unrelated families and seventeen sporadic cases) diagnosed with osteogenesis imperfecta type I-IV nineteen of these individuals are affected by OI type IA, five suffer from the third type of the disorder and one is diagnosed with OI type IVB. All of them signed an informed agreement permitting the molecular genetic analysis of their DNA. Blood samples were collected at several workplaces in the Czech Republic, such as Prague, Brno, Hradec Králové, Olomouc or Ostrava. Molecular-genetic analyses of the isolated gDNA were focused on the COL1A1 gene.

The gDNA was isolated by using the QIAamp DNA Blood Midi Kit (QIAGEN) and stored at -20°C. The quality of isolated samples was determined by gel electrophoresis and the quantity was detected spectrophotometrically.

Thus verified DNA samples were amplified using a polymerase chain reaction (PCR) and specially de-

Table 1: Overview of detected mutations/polymorphisms in Czech OI patients.

Patient No.	OI form	Gender	Age (years)	Nucleotide change	Mutation/ Polymorphism	COL1A1 position
1	III	Female	23	GGC/TGC	Gly526Cys	exon 31
2	IA	Male	22	ACT/ACC T/C C/G	Thr588Thr I32T15375C I39C17332G	exon 33 intron 32 intron 39
3	IVB	Female	52	ACT/ACC T/C C/G	Thr588Thr I32T15375C I39C17332G	exon 33 intron 39 intron 31

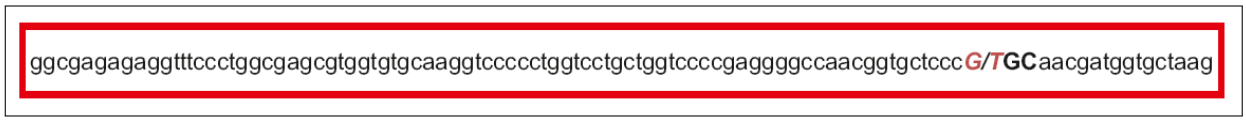

Figure 2: Sequence structure of the exon 31. Position of the Gly526Cys substitution is marked in bold, the changed nucleotide is marked in red italics.

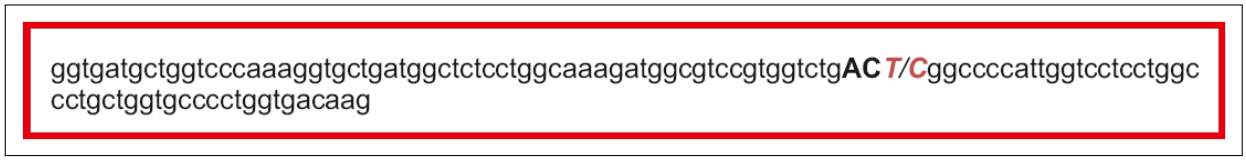

Figure 3: Sequence structure of the exon 33. Position of the Thr588Thr substitution is marked in bold, the changed nucleotide is marked in red italics.

signed 100% complementary primers focused to six regions (G1-G6) of the DNA involving exons 31 to 40. This section was chosen based on presence of the multi ligand binding region 2. The sequences of the used oligonucleotide primers are G1-1 CATCCGTCAAGGTGCGTCG and G1-2 CCTGCCCTGGTCTTTTCCC which amplify a 350bp long region including the exon 31; G2-1 CTGGAGTCTGGGCTGTGAG and G2-2 GTGTTCTGCTTGTGTCTGGG primers producing product with length of 660bp containing the exon 32; G3-1 CCAGACACAAGCAGAACACT and G3-2 CTGAGAGCAAGGGACAAGA focused on a 402bp long region including the exon 33; G4-1 TCAACCTGGGAACCTGGAG and G4-2 CAGCATCGCCTTTAGCACC that produce a 662bp long PCR product comprising exons 34 and 35; G5-1 TTCCTGCCTCCATTACTGC and G5-2 AACAGCCAACTCATCCGAC amplifying a 426bp long region with exons 36 and 37; and in conclusion primers G6-1 GGTGCTACTGGTTTCCCTGG and G6-2 TCTGTTCTCCTTGGCTCCGC defining a 645bp long DNA region containing exons 38, 39 and 40.

The polymerase chain reaction amplification was performed in 50 μl final volume, with 100 ng of genomic DNA, 25 μl Taq PCR MasterMix (1000U) (QIAGEN) (contains Taq polymerase (5 U/μl), PCR Buffer, $MgCl_2$ (1,5 mM), dNTPs (4 x 200 μM)) and 0,5 μl (50 pmol) of each of the oligonucleotide primers.

We performed 35 cycles of 0,5 min at 95°C, 0,5 min at 59°C (system G1)/ 58°C (systems G2, G4 and G6)/ 57°C (system G3) /53°C (system G5), and 1 min at 72°C. The amplified products were electrophoresed through a 2% agarose gel.

Sequencing of PCR-amplified COL1A1 gene fragments was carried out using an automatic capillary sequencing method. We used in this research BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corporation, USA) protocol.

Obtained data were compared with corresponding COL1A1 gene segments of the healthy population – for this analysis were used PC programs DNA Baser and SeqScape® Software for Mutation Profiling v2.7. Identified mutations were compared with the OMIM database.

3 Results

Molecular-genetic analyses revealed mutations in three of twenty-five analysed Czech OI patients. Changes of the DNA were found in both coding and noncoding regions of the COL1A1 gene namely in exons 31 and 33 and in introns 32 and 39 (Table 1, Figure 1).

We detected a substitution of glycine to cysteine at position 526 in the exon 31 (Figure 2) in the DNA sample from a patient affected by the third type of OI (section 3.1.1). This mutation is the most common described change within this form of the disorder. First time was this change described in 1989 by Starman et al. [14].

The second mutation of the coding sequence was identified in patients diagnosed with osteogenesis imperfecta type IA (section 3.1.2) and IVB (section 3.1.3). In both cases it is a silent mutation of threonine at position 588 in the exon 33 (Figure 3). In these patients were also detected noncoding sequences changes of introns 32 (Figure 4) and 39 (Figure 5).

3.1 Case Reports

3.1.1 Osteogenesis Imperfecta Type III

The first case is a 23 years old woman born from the second gravidity in the family with unaffected parents. Birth was performed by Caesarean section. The newborn was resuscitated. She weighed 2450 g, birth length was 45 cm. The diagnosis was confirmed immediately at the birth.

Clinical features presented in this individual are blue sclera, trigonocephaly, hyperbrachycephaly, wormian bones of the skull, moderate exophtalmos and hypermobile joints. The woman does not suffer from otosclerosis

Figure 4: Sequence structure of the intron 32. Position of the I32T15375C polymorphism is marked in red italics.

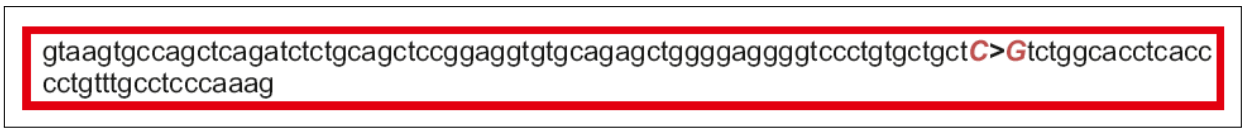

Figure 5: Sequence structure of the intron 39. Position of the I39C17332G polymorphism is marked in red italics.

and dentinogenesis imperfecta. Fractures of both femurs occurred during childbirth, X-rays showed up healed fractures of ribs and the left clavicle. The patient during childhood and adulthood suffered from multiple fractures especially of long bones of lower and upper extremities. The last fracture – fracture of the right clavicle, was described at the age of 19 years. She began to walk at six years with help of leg orthosis. She has used the wheelchair since 11 years of age. Radiological examinations of skeleton at the age of 15 and 18 year revealed suspicion on osteoporosis. Densitometric scans confirmed some decrease of bone mineral density (BMD). Anthropologic and X-ray examinations verified the presence of barrel chest with deformed ribs, pectus carinatum, severe scoliosis, platyspondyly of thoracic vertebras, higher bodies of lumbar vertebras, biconcave shape of thoracic and lumbar vertebras, deformation of skull bones, angulation of the right forearm and femurs, saber shaped deformities of humeri and tibias and shortening of femurs. Metaphyses and epiphyses of bones of the knees have popcorn-like structure, the typical radiological sign of the third type of this disorder (Figure 6).

Medical treatment namely with calcitonin has started at the age of seven years. Treatment with bisphosphonates has begun seven years later. A part of the medical treatment is supplementation with calcium and vitamin D3. The patient has undergone a lot of surgeries since 2nd year of age (corrective and multiple osteotomies with intramedullary nailing). Orthotic treatment was a part of comprehensive treatment since 6 to 16 years of life.

Molecular-genetic analyses identified the most typical mutation for this OI type – Gly526Cys. Further was at the patient identified mutation of MTHFR gene (heterozygous A1298C) increasing blood coagulation.

3.1.2 Osteogenesis Imperfecta Type IA

The second case of our report is a case of a 22 years old man affected with the 1st type of the disease. The patient was born from the third gravidity of unaffected couple. Birth weight was 2800 g, the birth length was 50 cm.

He has light blue sclera (Figure 7), suffers from hearing loss and tinnitus. On the skin of face, neck and chest are numerous lentigo. Other clinical signs include slim chest with narrow vertical ribs, high palate, weak muscles, hypermobility of joints and asymmetric shoulders. The first fracture occurred in the age of 2 years.

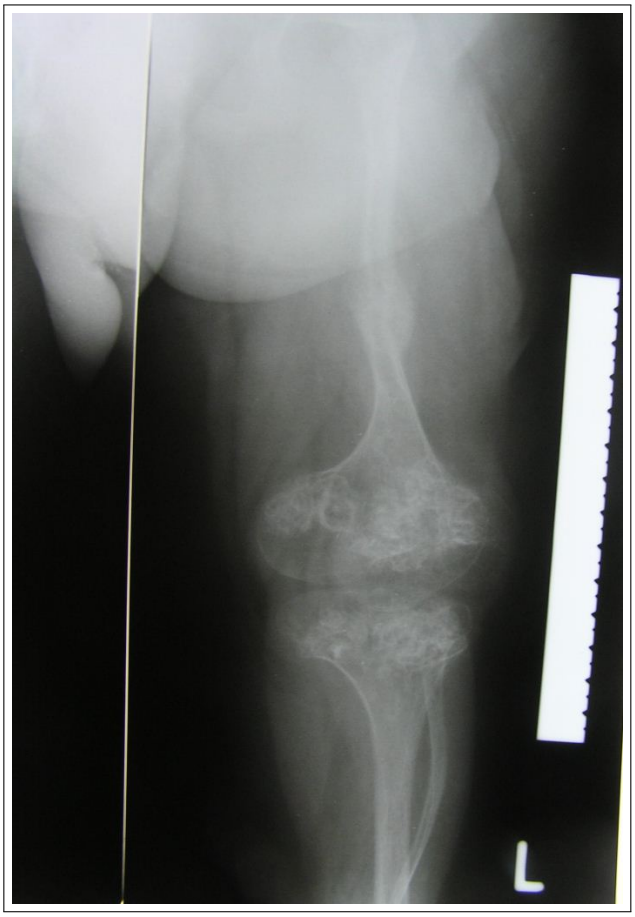

Figure 6: Popcorn-like structure of the femoral epiphysis of the patient suffering from OI type III.

Other fractures occurred at 9 years (fracture of the thoracic vertebrae), at 11 years (fractures of both ulna) at 15 years of life (fracture of the second metacarpal bone of the right hand). X-rays present deformities of the spine – straightened thoracic kyphosis, flattened lumbar lordosis, platyspondyly of thoracic vertebrae and moderate scoliosis. Long bones of the lower limbs are mild bowed and the patient has shortened fourth and fifth metatarsal and digit bones. Densitometric examination confirmed low bone mass according to chronologic age (Z-score is less than -2,0).

The patient is treated with bisphosphonates, calcium and vitamin D3. He has undergone many surgeries, such as incorporation of Kirschner´s rods and tympanostomy.

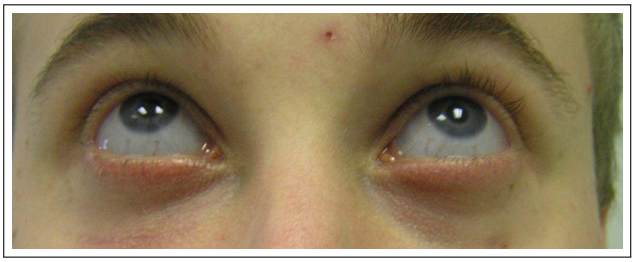

Figure 7: Light blue sclera of the patient affected by OI type IA.

3.1.3 Osteogenesis Imperfecta Type IVB

A 53 years old woman affected by the 4th type of osteogenesis imperfecta is the first child in healthy family without signs of increased bone fragility. Birth anthropometric parameters were 2840 g and 47 cm.

The patient has blue sclera, otosclerosis and dentinogenesis imperfecta - she lost her second dentice when she was 20 years old. She has generalized joint hypermobility, short body and lower limbs and suffers from back pain. The patient suffered multiple fractures especially of bones of lower limbs since she was 2 years old. The last fracture occurred at the age of 14 years. X-ray examinations demonstrate biconcave shape of thoracic and lumbar vertebrae bodies, saber deformities of tibias (Figure 8) and right femur, varus femoral necks and valgus heels. Densitometric examinations determine osteoporosis of the skeleton (T-score is less than -2,5).

Medical treatment with bisphosphonates has begun at 42nd year of age. She is further also treated with vitamin D3. The woman has undergone only one surgery when she was 47 namely of the left femur. Currently she uses wheelchair or crutches and a knee brace.

Other molecular-genetic analyses identified a heterozygous mutation of a MTHFR gene (A1298C) and homozygous mutation of a UGT 1A1 gene (7TA/7TA) that causes Gilbert syndrome.

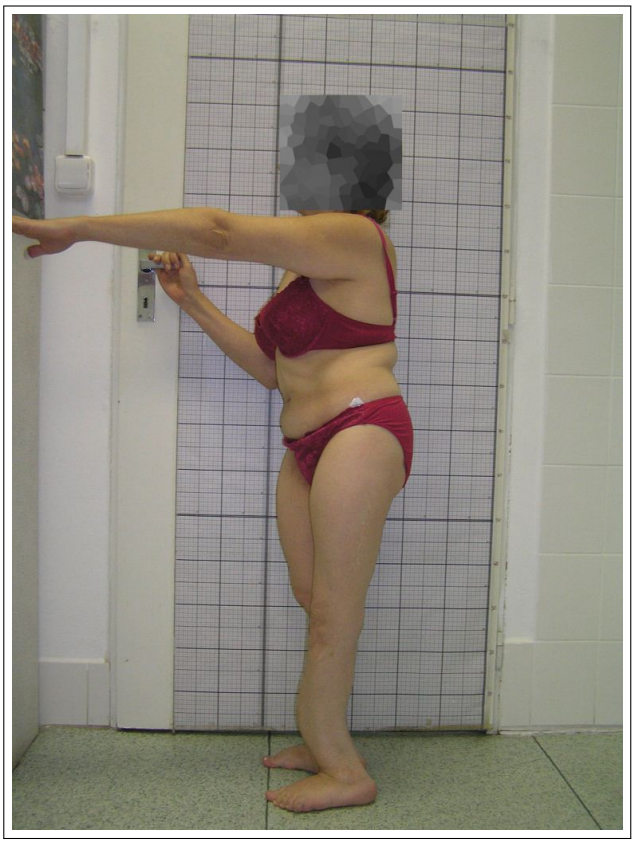

Figure 8: Saber deformity of the left tibia of the patient diagnosed with OI type IVB.

The family anamnesis in this case is interesting because the husband and daughter of this patient are affected by the Charcot Marie Tooth syndrome in combination with the diabetic neuropathy. Her daughter also suffers from muscles atrophy, cramps and paresthesia of lower limbs.

4 Discussion

Osteogenesis imperfecta is the highly heterogeneous disorder with molecular-genetic background in mutations especially of genes coding the collagen type I. The clinical picture of affected patients differs inter- and intra-group. Currently, world literature describes some relationships between positions of mutations and resulting phenotype of individuals. Generally, lethal phenotype results from mutations situated to the C terminus of alpha chains, substitutions of the glycine and substitutions by amino acids with branched side chains. It results further from mutations resulting in skipping of exons 3´ (especially exons 14, 20, 22, 27, 30, 44 and 47) of the COL1A1 and exons 5´ to the exon 27 of the COL1A2 gene) and from mutations resulting in creation of alternative or cryptic splice sites [8]. There are also two regions named MLBR2 and MLBR3 within the alpha1 chain and eight regions of the alpha2 chain whose mutations result namely in lethal OI types II or III. On the other hand, mutations of the first 200 amino acids, the glycine substitutions at the first 85-90

amino acids, nonsense mutations resulting in production of STOP codons and changes situated in the N terminal area of the alpha chains exhibit nonlethal clinical picture [4, 8]. In conclusion we can say that in general mutations of the COL1A1 gene usually display in more severe clinical features than these of the COL1A2 gene. But we should not forget that other factors such as genetic, nutrition or environmental changes may affect expression of mutations.

In this study we analyzed 25 Czech patients suffering from collagenous forms of OI. DNA defects were detected in three of these patients. These changes are in two cases novel single point mutations or polymorphisms.

The glycine substitution for cysteine at the position 526 was determined in the case of a woman diagnosed with OI type III. Starman et al. described this mutation in 1989 in an Iraqi individual. Both of these patients showed similar clinical signs such as deformation of bones, presence of wormian bones, fractures at birth, blue sclera and defective dentin production without dentinogenesis imperfecta [14]. This mutation is situated in the integrins binding region of the alpha chains. Variations of this area affect production of intermolecular and molecule-extracellular matrix linkages and decrease strength of bones. Because it is the most common substitution identified in OI type III patients we can conclude that it results in severe bone deformity.

The Thr588Thr mutation was identified in two patients suffering from different OI types – types IA and IVB. Despite this the patients have some of the identical features – blue sclera, hearing loss, hypermobility of joints and osteoporosis. Although the silent threonine 588 substitution does not alter the reading frame it can negatively affect translation parameters and production of intermolecular linkages with the COMP which binds to the collagen type I at the site defined by codons 582 to 638. We can consider that a silent mutation may predict development of osteopenia and osteoporosis due to change of one of some nucleotides in COMP binding site. However, this is only a speculation. Currently, literature does not describe this silent mutation.

Both of identified polymorphisms (I32T15375C, I39C17332G) were detected in patients with the same substitution Thr588Thr in exon 33. Any of these changes result neither to formation of STOP codons nor to the production of an extended/shortened product due to using of cryptic splice-sites. It follows that they do not result in defective production of the collagen type I. Currently any worldwide literature does not describe these polymorphisms.

5 Conclusion

We collect currently further biologic material such as venous blood, bone grafts or tissue of aborted embryos of the Czech patients affected by osteogenesis imperfecta type I-IV for other molecular genetic analyses focused on the other coding sequences of the COL1A1 gene. For next analyses we will use methods High Resolution Melting Analysis and the Sanger sequencing technology. This will be performed in cooperation with the Centre for Medical Genetics – University of Antwerp, Edegem, Belgium.

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