

Cell therapy of a patient with type III *Osteogenesis imperfecta* caused by mutation in *COL1A2* gene and unstable collagen type I*

Marcin Majka¹, Magdalena Janeczko², Jolanta Goździk³, Danuta Jarocha¹, Aleksandra Auguściak-Duma^{4,5}, Joanna Witecka^{4,5}, Marta Lesiak^{4,5}, Halina Koryciak-Komarska^{4,5}, Aleksander L. Sieron^{4,5#}, Jacek Józef Pietrzyk^{2#}

¹Department of Transplantation, UJCM, Krakow, Poland

²Department of Pediatrics and Medical Genetics, Chair of Pediatrics, UJCM, Krakow, Poland

³Department of Clinical Immunology, UJCM, Krakow, Poland

⁴Department of General and Molecular Biology and Genetics, Medical University of Silesia, Katowice, Poland

⁵Network of CoE BioMedTech, Poland

Email: #mipietrz@cyf-kr.edu.pl, alsieron@sum.edu.pl

Received 25 January, 2013; revised 28 February, 2013; accepted 10 March, 2013

ABSTRACT

The allogenic bone marrow derived mesenchymal stem cells transplantation was given to the newborn girl diagnosed with *osteogenesis imperfecta* type III, with multiple bone fractures, extreme shortness and limbs deformities. The treatment was performed at the age of 4 and 6 weeks. The clinical diagnosis was supported by biochemical analysis of collagen type I recovered from culture medium of cultivated patient's skin fibroblast, which revealed its triple helix instability at temperature about 2°C lower than normal. Sequencing of both genes encoding procollagen type I revealed heterozygous substitution G23569A in *COL1A2* gene causing change of glycine at position 517 to aspartate. The donor of mesenchymal stem cells was the girl's father. She received two intravenous infusions of suspended cultured mesenchymal cells in 16 days apart without any side effects. An analysis of procollagen type I secreted to the culture medium by bone marrow-derived mesenchymal stem cells obtained from the patient, 3 months following transplantation revealed its normal triple helix stability. During the subsequent two years of follow up two new bone fractures were noted. Currently a two-year-old girl's presents extreme growth and weight deficiency. The motoric development is also retarded, but the patient constantly improves and makes progresses.

Keywords: Bone Mineralisation; Cell Therapy; Collagen Type I; *Osteogenesis imperfecta*; Triple Helix Stability

*No conflict of interest exists for each author.

#Corresponding authors.

1. INTRODUCTION

Osteogenesis imperfecta (*OI*) is a genetic disease determined by autosomal dominant negative mutation of type I collagen, which is commonly known as "brittle bone disease". The overall birth prevalence of *OI* is estimated to range between 1 in 20,000 and 1 in 30,000. Clinically *OI* is heterogenous disorder with wide range of manifestations, from very mild, through moderate, to fatally severe, including lethal cases [1]. It is a systemic disorder affecting primarily bones but also teeth and eyes as well as leading to progressive hearing impairment [2]. Bones in this condition are fragile such that affected individuals sustain fractures after very mild trauma and frequent fractures, from several to tenths are observed throughout the patients' lives [3]. Multiple skeletal features and poor healing is responsible for short stature, relative macrocephaly, scoliosis and bowing of the long bones. Two types of the *OI* are represented by most severe clinical outcome [4]. The type II *OI*, which is the most severe form of *osteogenesis imperfecta* and is almost uniformly lethal. This form of the disorder is characterised by extreme bone fragility, leading to fetal demise or death in perinatal period [5,6]. Second one in severity of clinical symptoms is type III of *OI*, which is leading to serious skeletal deformities and makes the patient to be wheelchair or bed ridden. The frequency of type II and III *osteogenesis imperfecta* is estimated to be 1 in 10000.

The gold standard for postnatal diagnosis of *OI* is based on clinical and radiological features, confirmed subsequently by molecular diagnosis of collagen triple helix stability and detection of mutation [7]. In the majority of cases the cause of the *OI* is the mutation in

one of the two genes encoding procollagen type I polypeptide chains, namely *COL1A1* and *COL1A2*. In recent years, however, new evidence surfaced shading light on other genes, which when mutated might lead to autosomal recessive forms of *OI* although both procollagen genes do not show the mutation [8-11]. The incorrect genes both encoding procollagen type I and potential disturbances in its posttranslational processing are the major causes of poor quality of extracellular matrix, regardless lower collagen content or its poor stability.

Conventional management for patients with *OI* consisted of supplementation with vitamin D and minerals, or administration of bisphosphonates [8,12-15]. However, these methods did not ameliorate the symptoms of *OI* [12]. Moreover, in young teenage patients the bisphosphonates cannot be used continuously, because they slow down bone turnover and as consequence child's growth and healing of eventual bone fractures [16]. Another option of supportive treatment of *OI* patients is surgical and orthopedic intervention or protection with the use of braces, steel rods and other orthopedic equipment [reviewed in 11]. Either, supportive treatment of *OI* did not provide sufficient clinical improvement, especially in the most severe forms. Neither the medications or the surgical treatment can lead to permanent amelioration of the *OI* symptoms. The obvious reason is the genetic nature of this disorder.

For patients with *OI* type II and III a cell or gene therapy could be offered as the only chance of life saving and prevention of severe disability. Already more than 10 years ago first clinical trials were conducted for treating the severe type of *OI* with cell therapy [17]. The trials were then further expanded on larger group of *OI* patients [18-20].

Here, we report a case of a girl with *OI* type III treated at the age of 4 and 6 weeks by transplantation of allogeneic bone marrow derived mesenchymal stem cells (MSC). The diagnosis of *OI* was established on the base of clinical and radiological features, and confirmed subsequently by molecular diagnosis which revealed procollagen thermal instability and detected a missense mutation in *COL1A2* gene.

2. MATERIAL AND METHODS

2.1. Patient

A girl patient was born to a G2, P1 healthy mother at 39/40 weeks of gestation. A child was delivered by caesarean section, because of abnormal fetal heart rate pattern, pelvic position, and severe deformities of upper and lower extremities detected prior to the child's birth during regular monitoring of pregnancy by ultrasound examinations. Both parents are healthy and unrelated

individuals. The first pregnancy of this couple was a miscarriage. The analysis of family pedigree did not show any cases of *OI* or any other genetic diseases. The birth parameters of the patient were as follow: birth weight 2490 g, length 44 cm, head circumference 34 cm, Apgar score 3, 4, 5, 7 points. In delivery room the child was successfully resuscitated. The patient required support of ventilation (nasal CPAP) shortly after birth and during the first three days of life. Neither mechanical ventilation nor oxygen therapy were needed later on.

At the age of three days, the child was admitted to the Neonatal Intensive Care Unit of the Department of Pediatrics, Chair of Pediatrics, Jagiellonian University and hospitalized for subsequent three months. Clinical evaluation of the patient on admission revealed short stature, severe lower limb deformities, relative macrocephaly, triangular face and blue sclera. X-ray examination (baby-gram) showed 27 bone fractures, multiple deformities and reduced ossification of the skull calvarium. Based on the clinical and radiological findings of multiple bone fractures and severe limb deformities, the preliminary diagnosis of type II *OI* was made according to the classification by Silience [21]. The anthropometric measurements were conducted and the dermatoglyphic patterns were analyzed. Genomic DNA was isolated from cultured fibroblasts for the molecular analysis of the *COL1A1* and *COL1A2* genes. The skin biopsy was performed to assess the stability of produced by fibroblasts collagen triple helix. The levels of serum bone turn-over markers (osteocalcin (OC), procollagen type I N-propeptide (P1NP), collagen type I crosslinked C-telopeptide (Ctx)) were assessed. Then, on the basis of the natural cause of the disease, the result of molecular analysis revealing heterozygous mutation in exon 26 of *COL1A2* gene and the diminished thermal stability procollagen triple helix, the diagnosis was changed to type III *osteogenesis imperfecta*. The patient was qualified to a clinical trial entitled "Cell therapy in treatment of the patients with type II and type III *osteogenesis imperfecta*". The entry criteria for the study were as follow: 1) diagnosis of type II or III *osteogenesis imperfecta*, 2) parents agreement for participation in the clinical study, 3) approval of the Bioethics Committee of the Jagiellonian University (KBET/108/B/2007).

2.2. Patient's Samples

The anthropometric measurements were conducted and the dermatoglyphic patterns were analyzed before mesenchymal cells transplantation was performed. A blood samples were taken to assess the level of serum bone markers (OC, P1NP, and Ctx). The skin biopsy was performed to assess the stability of collagen triple helix and genomic DNA purification for molecular analyses of *COL1A1* and *COL1A2* genes.

2.3. Survey of Collagen Genes for Mismatches

DNA fragments of the collagen *COL1A1* and *COL1A2* gene were amplified using primers described previously [22] and listed in **Table 1**. The primers were designed and verified using PrimerSelect in DNASTar programme package version 5.0. The DNA was isolated from the proband's cultured fibroblasts, using BloodMini DNA purification kit (A & A Biotechnologies, Gdansk, Poland). The DNA was recovered from the kit column with 1 mM EDTA buffered with 10 mM Tris-HCl pH 8.5 at final volume 150 μ l and its concentration was determined spectrophotometrically at the wave length 260 nm and 280 nm. Quality of the DNA was verified following its electrophoresis in 0.8% agarose gel. Until further analyses the DNA samples were stored at -20°C . Concentrations of primers were calculated based on the sample absorbance at the wave length 260 nm and adjusted to the primer compositions. PCRs were performed according to the conditions described elsewhere [22]. Briefly, the reaction mixture included 300 ng double stranded patient genomic DNA, 1 μ l 10 mM dNTPs (PCR nucleotide Mix, Roche, Germany), 1 μ l (5 pmol) of each primer, 2.5 μ l 10x Taq polymerase buffer, 1U Taq polymerase

and nuclease free water (all from FastStart Taq DNA Polymerase, Roche, Germany) to the total volume of 25 μ l. The mixture was denatured for 5 minutes at 95°C , and incubated for 34 cycles of: 30 seconds at 95°C , 30 seconds at 60°C or 65°C , depending of the T_m of the specific pair of primers (**Table 1**), 30 seconds to 2 minutes at 72°C depending on the length of the amplified DNA fragment. When the last cycle was completed the samples were incubated for 7 minutes at 72°C then cooled down and left at 4°C . The PCR products (3 μ l) were separated on 1% agarose gel (Agarose, LE, Analytical Grade, Promega, USA) in TAE buffer.

2.4. PCR Conditions for DNA Sequencing

The PCR for sequencing was conducted according to recommendations provided by the DNA Analyser 3130 \times 1 ABI Prism, manufacturer (Applied Biosystems, USA). Briefly 10 to 20 ng of the PCR product was purified using Shrimp Alkaline Phosphatase (Fermentas, USA) and Exonuclease I, *E. coli* (Fermentas, USA). Clean product was then combined in semi-skirted 96-well PCR plate with 5 pM of primer, 8 μ l of the ready to use mixture provided by the manufacturer BigDye Terminator v3.1

Table 1. Sequences of primers used for fragment amplification and for sequencing of the *COL1A1* human gene.

Fragment Exon No. and position	Primers for fragment amplification (Amplified fragment length in bp)	Primers for sequencing
1		
420 - 522		
2		
1986 - 2180	M1f 305-F GACGGGAGTTTCTCCTCGGGGTC	M1f M2fk
3		
2322 - 2356	N3R 2927-R GCGCAAAAGAGCCTGATGTTA	GGGAGGGGCGGGAAGTGAAAAAT N3R
4		
2468 - 2494	[2622]	GCGCAAAAGAGCCTGATGTTA
5		
2585 - 2686		
6		
3409 - 3480	N5F 2831-F GTTTGTGACATGTGCTTATTAGT	
7		
3708 - 3752	2RA1 4049-R GCTGGGATTGAAGGGAAGAGGTAA	N6F AGTGCCGTCTTCTGCCTTCA 2RA1
8		
3911 - 3964	[1218]	
9		
4128 - 4181		
10		
4680 - 4733		
11		
4850 - 4903	N7F 3983-F GATTCATACTCCTTCTACAAAC	N7F N7R
12		
5234 - 5287	3RA1 5647-R CTGGCCAGTCCCTAGAGTTCCT	GGCACCATCCAAACCACTGAAA 3RA1 4FA1
13		
5376 - 5420	[1664]	GAGCCTGGCAGCCCTGGTGAAAAT
14		
5537 - 5590		
15		
5705 - 5749	4FA1 5379-F GAGCCTGGCAGCCCTGGTGAAAAT	
16		
5928 - 5981	5RA1 6436-R ACCAGGCTGTCCATCAGCAC	4FA1 5RA1
17		
6239 - 6337	[1057]	

Continued

	18		
	6426 - 6470		
	19	5FA1 6134-F	5FA1
5	6574 - 6672	CTGATCATTGCTCTCCTGTCCTGT	W9FA1
	20	4RA1 7332-R	ACACCCTCAGCCCCTCGTCTC
	6804 - 6857	CCCGGCCGCAAGGAGAGGTTAC	4RA1
	21	[1198]	
	7076 - 7183		
	22		
	7278 - 7331		
	23		
	7457 - 7554		
	24	6FA1 7166-F	6FA1
6	7721 - 7774	GGACCCCTGGCGAGCGTGTA	8FA1
	25	6RA1 9166-R	AGGCCCTGGCTTCACTTCA
	7863 - 7961	GGGGAGGCCGAGGACGAG	
	26	[2000]	
	8858 - 8911		
	27		
	9055 - 9108		
	28		
	9212 - 9265		
	29		
	9377 - 9430		
	30	W9FA1 9153-F	W9FA1
7	9882 - 9926	ACACCCTCAGCCCCTCGTCTC	M30F
	31	W9RA2 11221-R	GGTTCCTCTCTAATCACGGCCAGACC
	10020 - 10118	AGCCTCCCCTCCTTGGT	W8FA1
	32	[2068]	GCGGGGCTTAGGGCTGTGACC
	10416 - 10523		W9RA2
	33/34		
	10982 - 11089		
	35		
	11310 - 11363		
	36		
	11527 - 11580		
	37		
	11799 - 11906		
	38		
	11995 - 12048		
	39	9FA1 10501-F	W9RA3
8	12175 - 12228	GCTCCCATCTCTGCCTGCTTTGA	ACGCCTTGTCCTCATTC
	40	W10RA3 13247-R	W9RA4
	12369 - 12530	TGGCGGGGAGAGCAGGGGAATA	CCCACCCAGCACCCCAACCTA
	41	[2746]	W10FA2
	12632 - 12739		TGTTCTCCCTCTGACTGTTCCTAT
	42		W10RA3
	12897 - 13004		
	43		
	13112 - 13165		
	44		
	13270 - 13377		
	45		
	13758 - 13811		
	46	W10FA3 12800-F	W10FA3
9	13924 - 14031	GCGGAGCCAAGGAGAACAGAT	M46r
	47	W11RA3 15311-R	GGGAAAGAATGACTATCCAG
	14370 - 14423	GCATCATCAGCCCGGTAGTAGC	W11FA2
	48	[2511]	CCCCTGGCCCTCTGTAAGTATGC
	14784 - 14891		W11RA3
	49		
	14983 - 15265		
	50		
	15398 - 15588		
	51	N9F 14922-F	P48
10	15877 - 16119	GCCCTCAGCCCCATAGCACT	CAITGCCCTTCTGAGCACTGGGCTA
	52	N10R 16599-R	N10F
	16249 - 16395	GGTTTTGGTCATTGTTCCGGTTGGT	AGGTCCCTGCTAGTGGTTCA
		[1677]	M51F
			GGACCCTGGACAGGAGGCCAGCAGG

Cycle Sequencing Kit (Applied Biosystems, USA) containing polymerase, dideoxy-NTP in the buffer and the final volume was obtained by adjusting it with water up to 20 μ l. The mixture was incubated in the thermocycler by Eppendorf for 35 cycles with the following steps in the single cycle: denaturation for 30 seconds at 96°C, annealing 15 seconds at 50°C, elongation for 3 minutes at 60°C. After completing the last cycle the sample was cooled down to 4°C. The sequencing PCR product were purified by BigDye XTerminator Purification Kit (Applied Biosystems, USA) according to manufacturers manual and put in the instrument for the DNA sequencing. The sequences were compared with reference sequence (NG_007400.1 COL1A1 and NG_007405 COL1A2) using MegAlign in DNASTar programme package version 5.0. The differences were verified for final confirmation by analysis of the raw chromatogram sequencing data using Chromas Lite version 2.01 freeware.

2.5. Procollagen Type I Production and Purification

Procollagen type I was produced using skin fibroblasts prior and following the bone marrow mesenchymal stem cells transplantation. The skin biopsies of the size of 5 mm² were obtained using surgical blade. The sample was minced into small pieces and placed on a 100 mm in diameter Petri dish containing 10 ml of Dulbecco's Modified Eagle's Medium supplemented with 4.5 g/L glucose, (high glucose DMEM) 10% foetal calf serum (FCS), 10,000 units penicillin, 10 mg units streptomycin, 25 μ g/ml amphotericin B (all reagents from PAA The Cell Culture Company, Pasching, Austria). The medium was changed every other day. On day 7 of the culture the pieces of skin were removed and the fibroblasts were cultured until confluence. At 100% density the cells were transferred into T150 culture flask and maintained in the same medium until reaching 100% confluence when half of the culture was frozen, whereas, the other half was further cultured until 6 flasks of about 70% confluent were obtained. The procedure was followed for each sample. The fibroblast cultures were rinsed twice with sterile phosphate buffered saline (PBS) and filled with 15 ml of serum-free high glucose DMEM supplemented with ascorbic acid 50 μ g/ml and all the other supplements as described above. The medium was collected every day and replaced with the fresh one for three consecutive days. Then for 24 hours the culture was fed on high glucose DMEM with serum. The procedure of washing with PBS, harvesting the serum-free medium and feeding with total complete medium were repeated 4 times.

Harvested medium was processed as previously described [23] with subsequent modifications described elsewhere [22,24]. The volume of sample containing procollagen I was reduced to the appropriate collagen concentration using Amicon cell under nitrogen pressure

fitted with the membrane with the size cut off 100,000 kDa, following double dialysis against 200 volumes of so called storage buffer containing 0.4 M NaCl, 0.1 M Trizma Base (pH 7.4) supplemented with 0.025 M EDTA and 0.04% NaN₃.

2.6. Preparation of Collagen Type I and Its Triple Helix Thermal Stability Assay

The procollagen type I sample was treated with acetic acid to adjust pH to about 2.0. Pepsin was added to the acidified procollagen to a final concentration 200 μ g/ml. The digestion with pepsin was carried at 10°C for 18 hours, than terminated by raising pH to 7.5. The concentration of collagen was determined using kit by Biocolor (Ireland). Stability of collagen triple helix was assessed using J-815 Spectropolarimeter (Jasco) at 220 nm, using a 0.5 mm path-length thermostated cell. The temperature was controlled by a circulating water-bath. The temperature changes were controlled by a programmable controller (Jasco Peltier) which increasing temperature of collagen from 20°C to 45°C.

Stability of collagen triple helix was calculated by using Spectra Manager Program and Statistica 8.

2.7. Bone Mineralization Assay

Osteoblasts were detected by staining for the presence of hydroxyapatite in the culture using Alizarin Red-S (AR-S), following fixation in cold 70% ethanol at -20°C. At indicated osteoblast's differentiation time points, the medium was removed and the culture was washed with PBS followed by fixation in ice-cold 70% ethanol for at least 1 hour. Subsequently, the ethanol was removed, and fixed cells were rinsed with de-ionized water, then stained with 40 mM AR-S (pH 4.2) for 10 minutes at room temperature. After staining the cells were further processed by rinsing them five times with water followed by 15 minutes wash with PBS to remove nonspecifically bound AR-S. The specimens were photographed and immediately processed for minerals content. Therefore, bound AR-S was solubilized with 10% cetylpyridinium chloride (CPC) in 10 mM sodium phosphate, pH 7.0, for 15 minutes at 25°C. Recovered AR-S was diluted 10-fold in 10% CPC solution, and the AR-S concentration was determined by measuring the absorbance at 562 nm wave length using an AR-S standard curve in the same solution [25,26]. The values were normalized per 1000 cells. The measurements were conducted in quadruplicates.

2.8. Isolation of BM-MSCs

Bone marrow derived mesenchymal stem cells (BM-MSCs) were obtained from healthy patient's father after written consent according to the protocol approved by the Jagiellonian University Bioethical Committee (KBET/108/B/2007). Bone marrow (150 ml) was har-

vested under anesthesia from the posterior superior iliac crest by multiple aspirations. Aspirates were collected in a Bone Marrow Collection Kit with Pre-Filter and Inline Filters (Baxter, USA) and further processed. Light-density mononuclear cells (MNC) were separated on a Lymphocyte Separation Medium (PAA Laboratories GmbH, Goetzig, Austria) by density gradient centrifugation at 400 g for 30 minutes at room temperature. MNC were washed twice with Dulbecco's phosphate buffered saline (PAA Laboratories) and suspended in culture medium.

Culture of BM-MSCs

Bone marrow mononuclear cells were plated into vented 75 cm² tissue culture flask (Sarstedt, Newton USA) with Dulbecco's-Modified Eagles Medium (DMEM, Sigma-Aldrich Germany D5523) supplemented with 10% FBS (Stem Cell Technologies, Vancouver Canada 06472) and antibiotics (PAA Laboratories GmbH, Goetzig, Austria). Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and after 7 days medium was replaced with a fresh one. Then the cells were cultured with a medium change every week until confluence. On reaching confluence, the adherent cells were detached using 0.25% trypsin and re-seeded at 75 cm² flask (first passage). At the end of second passage when the cells reached confluence, they were trypsinized and checked for hematopoietic contamination [27].

2.9. Flow Cytometry

MSC phenotype was analyzed with mouse antibodies specific for human CD45 and CD3 antigens (Becton Dickinson). Briefly, to 1 × 10⁵ cells suspended in 100 μl of staining buffer (PBS, 2% FBS) 20 μl of mouse antibodies was added. Next, the cells were incubated in the dark for 30 minutes at 4°C. Stained cells were washed, collected and analyzed using FACS Calibur cytometer (Becton Dickinson, USA).

2.10. Infusion of the MSC

Cells were harvested after the second passage in two parts in two weeks time. Each time cells were suspended

in 10 ml of normal saline and passed through a filter before infusion. The patient received two 15- to 30-minute intravenous infusions of MSCs 16 days apart. Target doses for the first and second infusions were 4.0 × 10⁶ cells/kg of body weight and 3.9 × 10⁶ cells/kg, respectively. Directly before infusion the patient received hydrocortisone. The girl was evaluated for toxicity (physical examination, complete blood count, clinical chemistry panel-BUN, glucose, AST, ALT, ALKP, urine analysis, before, and 24 hours after, each infusion. She was monitored at the bedside during, and for 6 to 8 hours after, each infusion. Nine days after first infusion she received a course of the Vancomycine because of positive blood culture for Staph. Epidermidis. During the bacteremia period she was stable and in a good general status. The period of two weeks after the second infusion was uneventful. Then she developed G⁽⁻⁾ sepsis with *Enterobacter cloacae* treated with Meropenem and III generation cephalosporin antibiotics. She recovered from sepsis after two weeks and her general status was quite good.

Three months after MSC transplantation a bone marrow biopsy (for assessment the chimerism and cellular differentiation) and a skin biopsy (for evaluation the stability of collagen triple helix) were performed. Another blood sample was taken to molecular analysis of *COL1A1* and *COL1A2* genes.

The patient was finally discharged from hospital at the age of four months. She has had regular appointments in a genetic out-patient clinic (at the beginning every month, now every two months). Each visit consists of: history taking, physical examination, anthropometric measurements and collecting blood sample to assess the level of serum bone turn over markers.

3. RESULTS

3.1. Cells Expansion

Expansion of the cells resulted in harvesting 4 × 10⁶ cells and 3.9 × 10⁶ cells. Two infusions with collectively of 7.9 × 10⁶ MSC were performed. Expanded cells contained 7.3% of hematopoietic contamination, but did not contain lymphocyte contamination (**Figure 1**).

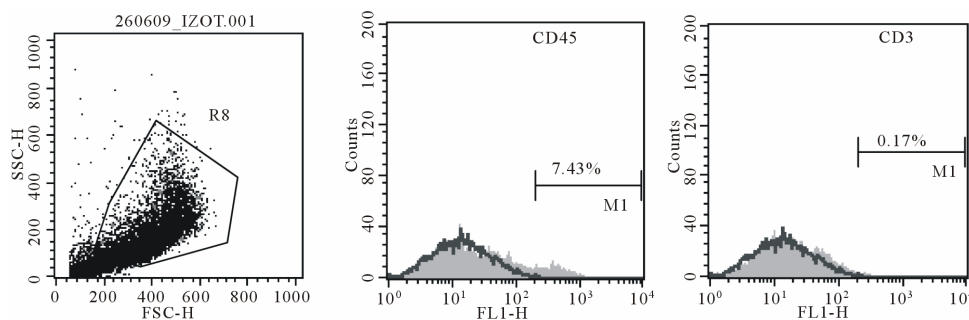


Figure 1. Majka M. *et al.* Cell therapy of a patient with type III osteogenesis imperfecta...

3.2. Patient Follow-Up

Patients follow up was performed monthly. Physical examination of the patient revealed short stature, severe lower limb deformities, relative macrocephaly, triangular face, blue sclera and *dentinogenesis imperfecta*. The antropometric measurements show extremely retarded growth (**Table 2**). The child's psychomotoric development was not synchronic—motoric development is retarded, while the speech development and cognitive functions are adequate to the chronological age. The patient is under the constant care of physiotherapist, orthopedic surgeon, psychologist and dentist. An objective audiology (ABR, AOE) examination was not possible to perform so far. Nevertheless, there is no signs of hearing loss in our patient.

The patient has had six bone fractures since her birth of which five were identified during the performance of diagnostic bone marrow biopsy after the MSC infusion. During the afterwards follow up over the period of two years two new fractures were diagnosed. The results of serum bone markers levels did not revealed any clinically significant tendencies.

Stability of collagen triple helix assessed in skin fibroblasts taken prior to bone marrow MSC transplantation showed temperature of 2°C lower than normal. Stability of collagen triple helix assessed in skin fibroblasts taken after the MSC transplantation returned to the normal temperature range (**Figure 2**).

The molecular analysis of the gene *COL1A1* performed prior to and following the MSC transplantation did not reveal any mutation. A missense mutation

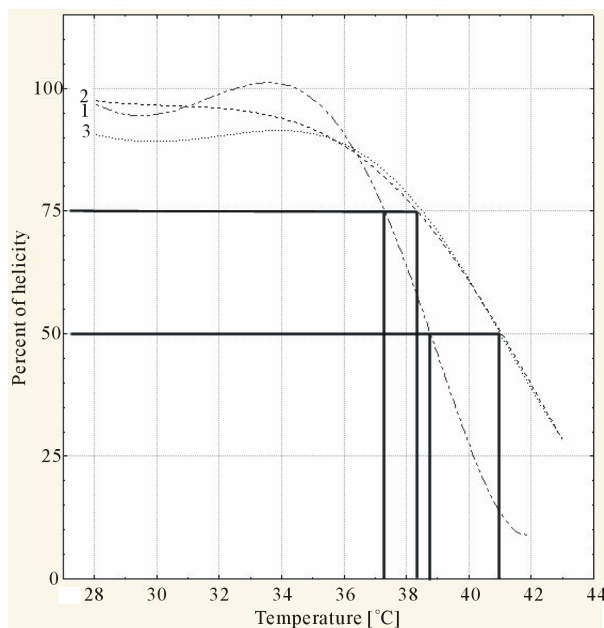


Figure 2. Majka M. *et al.* Cell therapy of a patient with type III *osteogenesis imperfecta*...

G23569A changing glycine at position 517 to aspartate (p.Gly517Asp) was detected in the exon 26 of *COL1A2* gene before and after the MSC transplantation. This particular mutation was previously identified in one patient with type III *osteogenesis imperfecta* reported to *Osteogenesis Imperfecta Variant Database*.

3.3. Bone Mineralization

Data on differentiation of bone marrow cells to osteoblasts measured indirectly as mineral deposition are inconclusive. Although the bone marrow MSC collected following transplantation could be successfully expanded during process of differentiation their number decreased with time. The experiments were repeated three times in quadruplicates and none of them was successful. The control sample in this experiment was established culture of stem cells purified from human normal placenta. The cells grew accordingly during differentiation and revealed increasing mineral deposition expressed as amount of calcium ion bound to AS-R (**Figure 3**).

3.4. Chimerism

Genetic profile of transplanted patients were studied using AmpFISTR SGM which recognized 11 STR loci. Only DNA of patient was detected indicating low engraftment of donor MSC.

4. DISCUSSION

Type III *OI* is known as progressive disease leading to serious skeletal deformations and severe handicap. Its presentation at birth is phenotypically similar to the milder end of the spectrum of type II disease. Although this form of the disorder is usually not life threatening, some children with type III *OI* die in infancy because of respiratory distress, whereas others die in childhood due to pneumonia, pulmonale or trauma. For those, who survive, there is progressive deformity of long bones and spine, which in combination with compression of vertebrae, contributes to marked short stature. Most of the patients with type III *OI* are severely physically handicapped and have traditionally required multiple orthopedic procedures and wheelchairs for mobility.

In fact, there is no effective treatment for the patients with *OI* so far [27-30]. That is why, it seems to be of crucial importance to continue research studies investigating the role of new methods of therapy in patients with this disease. A development of new treatment could improve the prognosis and reduce unnecessary exposure to potentially painful therapies.

We conducted a study examining the role of cell therapy in patients with type II and III *OI*. Till now, we enrolled one trial subject. MSC were administered

Table 2. Sequences of the primers used for fragment amplification and for sequencing of the *COL1A2* human gene.

Fragment Exon No. and position	Primers for fragment amplification (Amplified fragment length in bp)	Primers for sequencing
1	1 5742 - 5541	2FA2
	2 8188 - 8198	4FA2
2	3 8822 - 8836	TGCTGATCCCTGCCATACTTTTGAC JK3F
	4 9489 - 9524	3FA2 6944-F TTGAGAGTGGGAGGAAAGAGTCG JK3R
	5 10636 - 10728	N2RA2 11159-R TGTTATCTTAAACATCAAAGCTAC JK4F
		7FA2 N2RA2
3	6 12007 - 12060	7FA2 10526-F TCCACCCTACTTGCACATAGAAAGG 7RA2 13393-R
	7 16996 - 15040	JK6F TCGCCAAGTTTTTGACGTACAGCT JK6R
4	8 15133 - 15186	TGCGCTGGTAAAATGTGACATAAAA
	9 15279 - 15332	9FA2 JK10/11F
	10 15639 - 15692	ACCAAGATTCCCCCATTGTGCTGA 9RA2
	11 16113 - 16166	GAAGTCCAGTGTCCAGGTCCCAGC JK12R
	12 16690 - 16743	TGGAGGTCATGGGGAATTTCAATCA 11FA2
	13 18287 - 18331	
	14 18623 - 18676	JK14F CTTGTACAGGTTGGAAACTGAAC
	15 18776 - 18820	JK14R CCACGGGCACCCTAAGAAGA
	16 19210 - 19263	JK15F CCGTGGGCTTCCTGGTGAGAG
	17 19762 - 19860	JK16F CACCTGGATACCATGAATGTC
5	18 20004 - 20048	11RA2 TTCTTCTTCTCCCCATACTCCAC
	19 20163 - 20261	12FA2 JK18FB CGTTGGACCTCCTGTAAGTAG JK19F TAATGTGTGCTGCCTCTACAGC
	20 20682 - 20735	JK20F CTTGAGCTTCTCTTACCTTGAC
6	21 20860 - 20967	JK21F CGTAAGTAGCTCTATCATCAC
	22 21329 - 21382	JK22/23F GGGTGGGTGAAGTGTTTTGGCTTG
	23 21496 - 21594	JK23RB GCAACTGTCAGCAAGACTAC
7	24 22508 - 22561	13FA2 JK24F
	25 23024 - 23122	AAAAAGTCGGGGGAAAAGGTGCCTT JK25/26F

Continued

			TTTCATCCGTGGCAGCATCATAAGC JK25R CTGAGACTGGACTGATTCGCAG JK26R TATCAGATGGTGTAATAAAAAAAGTGTGGTTCT- TAGATG JK26FB TGGAGCTGCATGGTGATGGATC
	26 23523 - 23576	CAAAATGATATCAGATGGTGTAATA [1539]	
	27 24130 - 24183	14FA2 23556-F CTGGTCTTGCTGGTGCTCGGGTAG	14FA2 JK27/29F GCTTTCGTGGGAACCCACAATGAGT
8	28 24334 - 24387	14RA2 25707-R TGGACCAGCAGGACCAGGGAGACC [2151]	JK27RB TAGCAACGTATGTCACCACTG JK28RB TGCTTCAGTCTGAAATCATGT
	29 24662 - 24705	15FA2 24540-F GCTTAATAACATACAATCGTGCTC	15FA2 JK30F TGCACCTCATGTAGATACTGCCAGGT
9	30 25666 - 25110	15RA2 27582-R ACATCCTTCCATACACTAAAATAG [3042]	JK31F AAACCAGGGCTCGGAAGCTACACAA
	31 26845 - 26943		
	32 28164 - 28271	16FA2 27185-F GGTTGTCACCTTTTCTCCTCACG	JK32F TCTCCCTCCTTTCAATAGCCCAGCC
10	33 28939 - 28992	16RA2 30032-R TTTATAGATATTGCTGTTGGCTTAG [2847]	JK33F GAATGGTAAGGAATCGAGACATTGC JK34F GGAGTACCCTCCTCTGAGAGTGGC
	34 29938 - 29991		
	35 30673 - 30726		JK35F JK38F TGCGGGAATGATCCACTTGAAGAA
	36 30831 - 30884	JK35F 30535-F ACTCTGTGAGATGTGCGTCAG	JK36/37F GAAGCCCTGTAAGTAAAGAACCTG
11	37 30981 - 31088	JK40RB 33651-R CATCAACAAATAGATGCCACTTG [3116]	JK37R CTCCGTTATTTCCATCTTCTATC JK38R TCGGAATTGCTCTGAATAGAATGAA
	38 31449 - 31502		
	39 32339 - 32392		17FA2 JK39RB GAAAAGCTGACTTCAGACCAGGAG JK39FB GAAATTCCTCATCTTACCCAAATTCTTG
	40 33397 - 33558	17FA2 31958-F GGTCATTTGTTATTCTTTTGTCCTA N13RA2 35305-R GATGGTTTCTAGTTGTTGTCACG [3348]	JK40RB JK40FB CATAAAGGAAGACAGGAGTTGC 17RA2 CTCATTATTGTCTTCCCGATTG JK41F CTCACAAATCTCAAGCCAACCTGTG JK41R TCTGTACATTTGAAGTGCGACGCTT N13RA2
12	41 34776 - 34883		
	42 35557 - 35664		18FA2 JK43F AGGGTTCGTTACTGAGCACTG
	43 36050 - 36103	18FA2 35302-F TTTGGGATACTGAATGACACG	JK44F ATGGTCAACCCGGACACAAG
13	44 36190 - 36297	18RA2 36985-R CTACAGTTTACTTACAGCGATACC [1683]	JK45FB GTGGGCTTCACTTCTGACTTC
	45 36438 - 36491		
	46 36863 - 36970	N15FA2 25691-F GTGAAGCTGGTCTGATGTGAGTC	JK46F GCAGATTACCAGCAGAGGTGAGAGC
14	47 37448 - 37501	19RA2 38998-R TATTTAGCAGAAGGAGAGGTGTTT [3308]	JK47R GACACCAGGTACATGTGAGCTG JK47/48R TGGGGCTAACTTTAATGGGTTGTC
	48 37628 - 37735		

Continued

			JK49FB
49			GAACATGCTCCGTGTGAAGCTC
38067 - 38325			JK48F
			CAAGAGAAGACAGTTCATCTCTG
50			JK50FB
38733 - 38917	20FA2 38643-F		TGTTACTTATGAGAGTCAGTATCTTTC
	CTCTTCCTCTTAAATATGGGGTAG		JK51F
51	N16RA2 41458-R		CCCTTTTCCTAAGCTTGGATCTGAG
15	39628 - 39870	AAAAATACACCACGATAACAAC	JK51R
		[2815]	TTAACCCCTTTAGACCCCTTG
52			JK52FC
40687 - 40833			GGACAGACATCTTCAGAATGAC

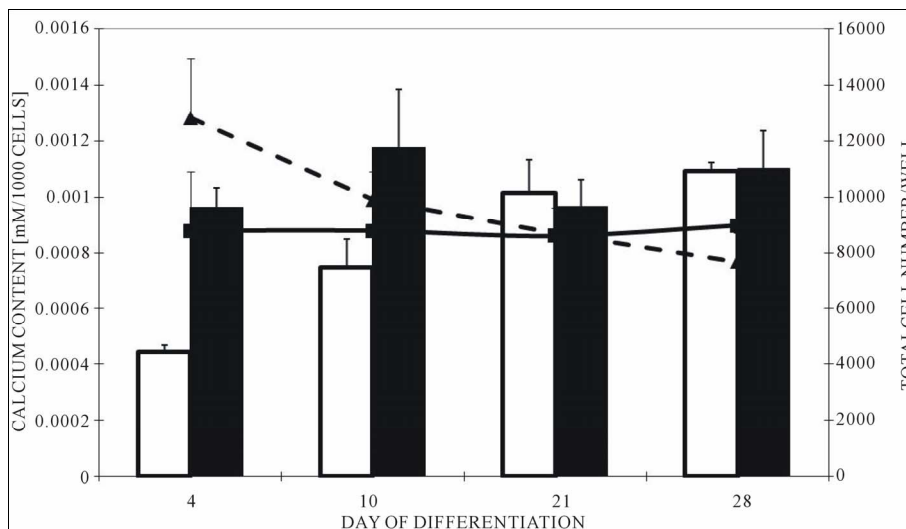


Figure 3. Majka M. *et al.* Cell therapy of a patient with type III *osteogenesis imperfecta*...

Intravenously. The most significant observation in the present study is the association between the MSC transplantation and the improvement of stability of collagen triple helix, and a decrease in a number of fractures observed in our patient. Stability of collagen triple helix after the MSC transplantation was normal, and there was only two bone fracture noted two years after the bone marrow stem cells transplantation. To validate this finding we measured serum bone markers levels, but they did not revealed any observable tendency. Nevertheless, during the first 2 years of life, the child had significant growth deficiency. Anyway, we can speculate that the cell therapy in patient with type III *osteogenesis imperfecta* looks promising. However, further evaluation is necessary.

Failure in detecting a chimerism in the patient could be a result that the threshold of the detection method used is 5%, thus, presence of donor cells could not be excluded if the cell engraftment fell under 5% and the donor DNA was not detected.

In summary, the stability of patient's collagen triple helix assessed in skin fibroblasts taken after the mesenchymal cells transplantation was within a normal temperature range. The girl has had only two bone fractures

two years after the transplantation. No cases of rejection reaction when the father's MSCs are injected into the girl's body were previously reported from other trials with the use of purified cells [17,18]. Therefore, in this study the graft versus host disease symptoms also were not observed. In conclusion, although, the assay of *in vitro osteogenesis* by minerals deposition was inconclusive, altogether the results indicate that they might be considered as an objective indicators of partial success of the cell therapy. It is very important for further clinical trials of cell therapy in the *OI* because no reports are available on remission of this disease, especially of its most severe type III.

5. ACKNOWLEDGEMENTS

The work was in part financed from Institutional grant KNW-1-010/P/1/0 and KNW-1-007/P/2/0 awarded to ALS. Some equipment used to conduct the research was co-financed by the EU funds (European Regional Development Fund) within the Sectoral Operational Program "Increase of Economic Competitiveness".

No. WKP1/1.4/3/2/2005/103/223/565/2007/U and Silesian Bio-Farma Center for Biotechnology, Bioengineering and Bioinformatics, Project no POIG.02.01.00-00-166/08, THE OPERATIONAL PROG-

RAMME INNOVATIVE ECONOMY FOR 2007-2013, Priority Axis 2. R & D Infrastructure.

REFERENCES

- [1] Rauch, F. and Glorieux, F.H. (2004) *Osteogenesis imperfecta*. *Lancet*, **363**, 1377-1385. [doi:10.1016/S0140-6736\(04\)16051-0](https://doi.org/10.1016/S0140-6736(04)16051-0)
- [2] Plotkin, H. (2004) Syndromes with congenital brittle bones. *BMC Pediatrics*, **4**, 16. [doi:10.1186/1471-2431-4-16](https://doi.org/10.1186/1471-2431-4-16)
- [3] Glorieux, F.H. (2008) *Osteogenesis imperfecta*. *Best Practice & Research Clinical Rheumatology*, **22**, 85-100. [doi:10.1016/j.berh.2007.12.012](https://doi.org/10.1016/j.berh.2007.12.012)
- [4] Van Dijk, F.S., Pals, G., Van Rijn, R.R., Nikkels, P.G. and Cobben, J.M. (2010) Classification of *osteogenesis imperfecta* revisited. *European Journal of Medical Genetics*, **53**, 1-5. [doi:10.1016/j.ejmg.2009.10.007](https://doi.org/10.1016/j.ejmg.2009.10.007)
- [5] Hackley, L. and Merritt, L. (2008) Osteogenesis imperfecta in the neonate. *Advances in Neonatal Care*, **8**, 21-30. [doi:10.1097/01.ANC.0000311013.71510.41](https://doi.org/10.1097/01.ANC.0000311013.71510.41)
- [6] Morgan, J.A. and Marcus, P.S. (2010) Prenatal diagnosis and management of intrauterine fracture. *Obstetrical & Gynecological Survey*, **65**, 249-259. [doi:10.1097/OGX.0b013e3181dbc50b](https://doi.org/10.1097/OGX.0b013e3181dbc50b)
- [7] Tedeschi, E., Antoniazzi, F., Venturi, G., Zamboni, G. and Tatò, L. (2006) *Osteogenesis imperfecta* and its molecular diagnosis by determination of mutations of type I collagen genes. *Pediatric Endocrinology Reviews*, **4**, 40-46.
- [8] Burnei, G., Vlad, C., Georgescu, I. and Gavrilu, T.S. (2008) *Osteogenesis imperfecta*: Diagnosis and treatment. *Journal of the American Academy of Orthopaedic Surgeons*, **16**, 356-366.
- [9] Basel, D. and Steiner, R.D. (2009) *Osteogenesis imperfecta*: Recent findings shed new light on this once well-understood condition. *Genetics in Medicine*, **11**, 375-385. [doi:10.1097/GIM.0b013e3181a1ff7b](https://doi.org/10.1097/GIM.0b013e3181a1ff7b)
- [10] Marini, J.C., Cabral, W.A., Barnes, A.M., Chang and W. (2007) Components of the collagen prolyl 3-hydroxylation complex are crucial for normal bone development. *Cell Cycle*, **6**, 1675-1681. [doi:10.4161/cc.6.14.4474](https://doi.org/10.4161/cc.6.14.4474)
- [11] Tarnowski, M. and Sieroń, A.L. (2008) *Osteogenesis imperfecta-etiology*, characteristics, current and future treatment. *Wiadomosci Lekarskie*, **61**, 166-172.
- [12] Dent, C.E. and Davies, I.J. (1980) Calcium metabolism in bone disease: Effects of treatment with microcrystalline calcium hydroxyapatite compound and dihydrotachysterol. *Journal of the Royal Society of Medicine*, **73**, 780-785.
- [13] Bachrach, L.K. and Ward, L.M. (2009) Clinical review 1: Bisphosphonate use in childhood osteoporosis. *The Journal of Clinical Endocrinology & Metabolism*, **94**, 400-409. [doi:10.1210/jc.2008-1531](https://doi.org/10.1210/jc.2008-1531)
- [14] Russell, R.G. (2007) Bisphosphonates: Mode of action and pharmacology. *Pediatrics*, **119**, 150-162. [doi:10.1542/peds.2006-2023H](https://doi.org/10.1542/peds.2006-2023H)
- [15] Benhamou, C.L. (2007) Effects of osteoporosis medications on bone quality. *Joint Bone Spine*, **74**, 39-47. [doi:10.1016/j.jbspin.2006.06.004](https://doi.org/10.1016/j.jbspin.2006.06.004)
- [16] Rauch, F. and Glorieux, F.H. (2005) Bisphosphonate treatment in *osteogenesis imperfecta*: Which drug, for whom, for how long? *Annals of Medicine*, **37**, 295-302. [doi:10.1080/07853890510007386](https://doi.org/10.1080/07853890510007386)
- [17] Horwitz, E.M., Prockop, D.J., Fitzpatrick, L.A., *et al.*, (1999) Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with *osteogenesis imperfecta*. *Nature Medicine*, **5**, 309-313. [doi:10.1038/6529](https://doi.org/10.1038/6529)
- [18] Horwitz, E.M., Prockop, D.J., Gordon, P.L., *et al.*, (2001) Clinical responses to bone marrow transplantation in children with severe *osteogenesis imperfecta*. *Blood*, **97**, 1227-1231. [doi:10.1182/blood.V97.5.1227](https://doi.org/10.1182/blood.V97.5.1227)
- [19] Pochampally, R.R., Horwitz, E.M., DiGirolamo, C.M., Stokes, D.S. and Prockop, D.J. (2005) Correction of a mineralization defect by overexpression of a wild-type cDNA for *COL1A1* in marrow stromal cells (MSCs) from a patient with *osteogenesis imperfecta*: A strategy for rescuing mutations that produce dominant-negative protein defects. *Gene Therapy*, **12**, 1119-1125. [doi:10.1038/sj.gt.3302514](https://doi.org/10.1038/sj.gt.3302514)
- [20] Niyibizi, C. and Li, F. (2009) Potential implications of cell therapy for *osteogenesis imperfecta*. *International Journal of Clinical Rheumatology*, **4**, 57-66.
- [21] Sillence, D.O., Senn, A. and Danks, D.M. (1979) Genetic heterogeneity in *osteogenesis imperfecta*. *Journal of Medical Genetics*, **16**, 101-116. [doi:10.1136/jmg.16.2.101](https://doi.org/10.1136/jmg.16.2.101)
- [22] Witecka, J., Auguściak-Duma, A.M., Kruczek, A., *et al.*, (2008) Two novel *COL1A1* mutations in patients with *osteogenesis imperfecta* (OI) affect the stability of the collagen type I triple-helix. *Journal of Applied Genetics*, **49**, 283-295. [doi:10.1007/BF03195625](https://doi.org/10.1007/BF03195625)
- [23] Fertala, A., Sieron, A.L., Ganguly, A., *et al.* (1994) Synthesis of recombinant human procollagen II in a stably transfected tumor cell line (HT-1080). *Biochemical Journal*, **298**, 31-37.
- [24] Arnold, W.V., Fertala, A., Sieron, A.L., *et al.* (1998) Recombinant procollagen II: Deletion of D period segments identifies sequences that are required for helix stabilization and generates a temperature-sensitive N-proteinase cleavage site. *The Journal of Biological Chemistry*, **273**, 31822-31828. [doi:10.1074/jbc.273.48.31822](https://doi.org/10.1074/jbc.273.48.31822)
- [25] Stanford, C.M., Jacobson, P.A., Eanes, E.D., Lembke, L.A. and Midura, R.J. (1995) Rapidly forming apatitic mineral in an osteoblastic cell line (UMR 106-01 BSP). *The Journal of Biological Chemistry*, **270**, 9420-9428. [doi:10.1074/jbc.270.16.9420](https://doi.org/10.1074/jbc.270.16.9420)
- [26] Gregory, C.A., Gunn, W.G., Peister, A. and Prockop, D.J. (2004) An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Analytical Biochemistry*, **329**, 77-84. [doi:10.1016/j.ab.2004.02.002](https://doi.org/10.1016/j.ab.2004.02.002)
- [27] Marini, J.C., Letocha, A.D. and Chernoff, E.J. (2005) *Osteogenesis imperfecta*. In: Cassidy, S.B and Allanson, J.E., Eds., *Management of genetic syndromes*. Published Online: 14 JAN 2005.

- <http://onlinelibrary.wiley.com/doi/10.1002/0471695998.mgs034/abstract>
- [28] Esposito, P. and Plotkin, H. (2008) Surgical treatment of *osteogenesis imperfecta*: Current concepts. *Current Opinion in Pediatrics*, **20**, 52-57.
[doi:10.1097/MOP.0b013e3282f35f03](https://doi.org/10.1097/MOP.0b013e3282f35f03)
- [29] Marini, J. (2010) *Osteogenesis imperfecta*. In: Diseases of bone and calcium metabolism. South Dartmouth in US: Mtext.com.inc.
<http://www.endotext.org/parathyroid/parathyroid17/parathyroidframe17.htm>
- [30] Chen, H. (2006) *Osteogenesis imperfecta*. In: Chen, H., Ed., *Atlas of genetic diagnosis and counselling*, Humana Press Inc., New Jersey, 762-772.