Mutations in the COL1A1 and COL1A2 genes associated with osteogenesis imperfecta (OI) types I or III

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Although over 85% of osteogenesis imperfecta (OI) cases are associated with mutations in the procollagen type I genes (COL1A1 or COL1A2), no hot spots for the mutations were associated with particular clinical phenotypes. Eight patients that were studied here, diagnosed with OI by clinical standards, are from the Polish population with no ethnic background indicated. Previously unpublished mutations were found in six out of those eight patients. Genotypes for polymorphisms (Sp1 – rs1800012 and PvuII – rs412777), linked to bone formation and metabolism were determined. Mutations were found in exons 2, 22, 50 and in introns 13 and 51 of the COL1A1 gene. In COL1A2, one mutation was identified in exon 22. Deletion type mutations in COL1A1 that resulted in OI type I had no effect on collagen type I secretion, nor on its intracellular accumulation. Also, a single base substitution in I13 (c.904-9 G>T) was associated with the OI type I. The OI type III was associated with a single base change in I51 of COL1A1, possibly causing an exon skipping. Also, a missense mutation in COL1A2 changing Gly→Cys in the central part of the triple helical domain of the collagen type I molecule caused OI type III. It affected secretion of the heterotrimeric form of procollagen type I. However, no intracellular accumulation of procollagen chains could be detected. Mutation in COL1A2 affected its incorporation into procollagen type I. The results obtained shall help in genetic counseling of OI patients and provide a rational support for making informed, life important decisions by them and their families.

Key words: osteogenesis imperfecta, COL1A1, COL1A2, mutation, polymorphism

Received: 17 May, 2017; revised: 13 February, 2018; accepted: 05 March, 2018, available on-line: 15 March, 2018

INTRODUCTION

Osteogenesis imperfecta (OI) is a heterogenic, connective tissue related disorder, manifested by bone fragility, with autosomal dominant, rarely recessive, inheritance and 1/10⁴ to 1/3 × 10⁵ prevalence (Roughley et al., 2003; Tarnowsk & Sieron, 2008; Forlino et al., 2011; van Dijk & Silence, 2014). The OI patients have poor quality of life due to frequent bone fractures, resulting from minimal trauma during regular daily activities. International Nomenclature Group for Constitutional Disorders of the Skeleton has proposed in 2009 classification of OI types based on radiological and clinical criteria (Silence & Rimoin, 1978; van Dijk et al., 2011; Warman et al., 2011). At present, there are 15 types of OI in OMIM. In this work we focused on patients with OI types I and III. The type I is the least severe (blue sclera, low bone fracture frequency and slight or no skeletal deformities) (Silence & Rimoin, 1978). Type III is an opposite progressive dominant form (Silence & Rimoin, 1978; Silence et al., 1979; Warman et al., 2011). In type III, in most severe cases the patients may develop respiratory problems leading eventually to death. Those patients which survive present severe health problems affecting their life quality (Tarnowski & Sieron, 2008; van Dijk & Silence, 2014).

About 90% of OI cases are due to causative variants in COL1A1 and COL1A2 genes resulting in abnormal collagen I fibrils formation (Forlino et al., 2011). The remaining 10% associate with recessive variants of 19 known or yet to be discovered genes (Dalgleish, 1997; Dalgleish, 1998; Galicka, 2012). Heterogeneity of OI symptoms and causative gene variants imposes complex modifying factors: genetic, epigenetic and environmental. Polymorphisms in COL1A1 and COL1A2 are among the factors influencing individuals to cope differently with mutational effects at the molecular level. Recently, more than 50 polymorphisms were identified in both the genes in the Indian population (Stephen et al., 2014). Two most common polymorphisms associated with diminished bone mineralization and lower resistances to trauma causing fractures are Sp1 in COL1A1 (Grant et al., 1996; Dehghan & Pourahmad-Jaltaji, 2015), and PvuII at position g.23065 in exon 25 of COL1A2. Sp1 is linked to low bone mineral density (BMD), osteoporosis, higher risk of bisphosphonate induced osteonecrosis of the jaw and an elevated risk of developing inguinal hernias (Katz et al., 2011; Kurt-Sirim et al., 2014; Sezer et al., 2014). The PvuII polymorphism is linked to variations in BMD, e.g. in pre-pubertal girls a transversion A→G in g.23065 (“p” allele) is strongly correlated with fracture frequency and variable bone strength parameters (Blades et al., 2010). In a dedicated database, Osteo-
genesis Imperfecta Variant Database there are 86 total public entries for COL1A1 and 58 total public entries for COL1A2 for Caucasian – Polish ethnic origin. No extensive comparison of both SNPs’ frequency in neither Polish nor in general Caucasian population is available. Therefore, the two polymorphisms already linked to bone density but not to OI, were also analyzed along with the mutations in procollagen type I encoding genes. Therefore, the aim of this work was to verify if there is a link between the clinical diagnosis of the OI type, the mutation detected in the affected patient and the individual status for the two SNPs, Sp1 and PvuII.

MATERIALS AND METHODS

Patient data. Clinical characteristics were collected by physician specialists in medical genetics who also co-author this report. The patient’s medical and family genetic histories were prepared based on “Clinical check list” according to EMQN guidelines for the diagnosis of OI patient, which for our patients was translated to Polish.

Methods

Materials. DNA was isolated from blood samples or skin fibroblasts obtained from five females (F) and three males (M) diagnosed with OI types I or III (Table 1). The study protocol was approved by the Bioethical Committee of Medical University of Silesia in Katowice, Poland (Approval No. KBET/356/B/2003).

Isolation and purification of genomic DNA. DNA isolation from blood or skin fibroblasts was conducted using DNA purification kit “Blood Mini” (A&A Biotechnology, Poland).

Conditions for amplifying and DNA sequencing PCRs. The PCRs were conducted using FastStartTaq DNA Polymerase kit (Roche, Germany) and the amplicons were verified. The reaction mixtures were prepared as described elsewhere (Witecka et al., 2008; Majka et al., 2013). The amplicons were verified following electrophoresis in 1% agarose gel using 5 µl of the obtained PCR products.

Sanger’s enzymatic method was used for DNA sequencing, therefore, 20 ng of each purified PCR product was mixed with 5 pM of the appropriate sequencing primer, for SpI: 5’-TCT-GGG-GAG-CCG-CTA-GCG-CGG-3’; for PvuII: 5’-TTT-CAT-CCG-TGG-CAG-CAT-CAT-AAG-C-3’ with Big Dye Terminator v.3.1. The sequencing PCR was conducted according to a protocol described elsewhere (Witecka et al., 2008; Majka et al., 2013).

Purified PCR sequencing products were analyzed using genetic analyzer ABI 3130xl (Applied Biosystems, USA) and the sequences were verified using Chromas Lite 2.01 and Nucleotide Blast (NCBI) software online to reference sequences NG_007400.1 for COL1A1 and NG_007405.1 for COL1A2.

Fibroblast culture, procollagen type I separation and SDS PAGE/western blot analysis. Fibroblast cells were obtained from skin biopsies as previously described (Witecka et al., 2008). The cells were cultured in T75 (75 cm²) culture flasks in Dulbecco’s Modified Eagle’s Medium supplemented with glucose (4.5 g/L), fetal calf serum (10%), penicillin (10000 IU/mL), streptomycin (10 mg/L), amphotericin B (25 mg/mL) (all reagents from PAA Laboratories, GmbH). The cells were cultured until reaching 80% of confluence. Then the cell monolayer was washed twice with sterile PBS and supplemented with medium free of antibiotics and serum. Following
| ID     | Age | Gender | Consanguinity | Family History | Diagnosis | Skeletal Problems | Other Physical Findings | Genetic Findings |
|--------|-----|--------|---------------|----------------|-----------|------------------|------------------------|-----------------|
| 55/F/41| 3   | N.D.   | N.D.          | Not reported    | Normal    | Blue             | YES (no description)   | Unspecified type of OI |
|        |     |        |               | Partial hearing loss on one side | NO | N.D. | YES | Son, OI type I; Daughter, OI type I |
| 56/F/8 | 4   | N.D.   | 4             | Not reported    | Correct   | Short stature 5%  | N.D. NO Blue NO N.D. NO OI diagnosis | Mother, OI type B; Brother, OI type I |
| 57/M/11| 3   | N.D.   | 3             | Not reported    | Correct   | Normal unspeci-fied | Blue NO N.D. YES OI diagnosis | Sister, OI type I |
| 73/F/11| 9   | 0      | 9             | Fully independent | No other problems except bone fractures | NO | Normal NO Blue NO YES (no description) | OI diagnosis None |
| 91-D/F/34| 20 | 0     | 20 last at 16 years of age | Partial loss on both sides | Normal NO Blue NO YES (no description) NO Unspecified type of OI | Son diagnosed with OI type I |
| 137/M/neonate| 11 | 11     | N.D.          | Infant difficult self-breathing (respiratory dyspnea) | Narrow rib cage, abnormal positioning of limbs, soft skull bones | 16 percentile Short Blue/gray N.A. YES (no description) YES Prenatally postulated OI type II/III | None |
| G9/M/9 | 3   | 0      | 3             | Low bone density | NO | An ankle valgus, shortened left leg, triangle face, Scoliosis, mild vertebral compression fractures, ptosis | Blue YES YES (no description) Not reported Postulated OI type III | N.D. |

N.D., No Data; N.A., Not Applicable
subsequent 48 hours, the medium was harvested, supplemented with the mixture of protease inhibitors in SigmaFast Protease Inhibitor Cocktail Tablet (Sigma, Germany) and divided in to two parts. One part was mixed with 0.25 volume of 5 × storage buffer to provide a final concentration of 25 mM EDTA and 0.04% NaN3 in 0.1 M Tris/HCl buffer (pH 7.4). The samples were filtered through a 1.6 mm glass-fiber filter (Millipore). Proteins from one part were precipitated overnight at 4°C with 176 mg/ml (NH4)2SO4. The precipitate was separated by centrifugation at 15000 × g for 30 minutes.

The other part of the medium was precipitated with 30% polyethylene glycol (PEG) at its final concentration of 5%. The precipitated proteins were recovered by centrifugation as from the previous part and both parts were stored at −20°C until their further analysis by SDS PAGE, followed by electro-transfer to Immobilon Transfer Membrane (Millipore, USA) and western blot analysis.

Simultaneously, fibroblasts used for procollagen production were detached using 0.25% trypsin in 1 mM EDTA (PAA Laboratories, GmbH). The trypsin and EDTA were neutralized with an equal volume of serum free culture medium and the fibroblast numbers were determined in samples pre-stained with Trypan Blue Dye, (0.4%) using TC20 Automated Cell Counter (Bio-Rad). Cold lysis solution (0.1% NP40 and 10µg/ml DNase I in PBS) was used at the volume enabling to obtain the fibroblast density of 20000 cells per 1 µl.

Proteins precipitated with ammonium sulfate were solubilized in a storage buffer (0.4 M NaCl, 0.1 M TRIS-HCl, 0.025 M EDTA, 0.04% NaN3; pH 7.4). The proteins, precipitated with PEG, were solubilized in 1 × SB buffer (0.25 M Tris-HCl, pH 6.8; 50% glycerol, 5% SDS, 0.05% bromophenol blue, 0.1% β-mercaptoethanol) in a buffer equal to the volume of solution used for lysis of fibroblasts recovered from the culture (all reagents were purchased from Sigma-Aldrich Co.). Total protein concentrations were determined using a spectrophotometer (Nanodrop2000, ThermoScientific, USA) and divided in to two parts. One part was mixed with the mixture of protease inhibitors in Sigma Fast Protease Inhibitor Cocktail Tablet (#C2456, Sigma).

RESULTS

Patients’ characteristics

The patient’s clinical phenotypes are summarized in Table 1. Six patients were clinically diagnosed as type I OI and two as type III. The initial diagnoses of five patients were without specification of the type of OI due to inconsistent examination protocols in different medical institutions (Table 1). In such situations, further examinations and additional medical tests were performed for complete clinical diagnosis of the type of OI (Table 2). For the three patients with OI type recognized initially, had their clinical diagnosis confirmed. Finally, four women were diagnosed with type I and one female was diagnosed with type III. In addition, the males had their OI confirmed as type I and III. Our observations indicate that a full panel of recommended clinical tests and examinations is required for complete diagnosis of the OI type. Particularly, X-rays of skeleton that in our study were available only for some of the patients, are necessary for correct diagnosis of the OI type (Fig. S1 at www.actabp.pl).

New mutations detected in COL1A1 and COL1A2 DNA sequences

An analysis of chromatograms obtained by sequencing of the PCR products revealed six heterozygous sequence changes (Table 2). Subsequent alignment of the sequences with respective reference sequences for COL1A1 and COL1A2 revealed three mutations in COL1A1 gene in four females and in one male, and two other mutations in two males (Table 2). In the COL1A2 gene, one previously unreported mutation was found (Table 2). At least one, but possibly three mutations, two in COL1A1 and one in COL1A2 genes, were inherited based on the family genetic history, whereas other mutations were detected in unrelated patients, without a family genetic history resulting in the production of an abnormal protein.

| Table 2. Summary of clinical diagnosis and DNA sequencing results of OI patients participating in this study. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **No.** | **Order** | **Patient’s No./Gender** | **Confirmed type of OI** | **Mutated gene** | **Location of mutation (E-exon; 1-Intron)** | **Position in cDNA** | **Mutation at protein level** | **Genotype of Sp1 polymorphism (rs1800012)** | **Genotype of PruII polymorphism (rs412777)** |
| 1 | 1 | 91-D/F | I | COL1A1 | E2 | c.231delG | p.Thr76Pro/ fs*76 | SS (G/G) | Pp (A/C) |
| 2 | 2 | G9/M | I | COL1A1 | I13 | c.904-9 G>T | not applicable | SS (G/G) | PP (A/A) |
| 3 | 3 | 55/F | I | COL1A1 | E22 | c.1503delT | p.Ala502Leu/ fs*5 | SS (G/G) | Pp (A/C) |
| 4 | 4 | 56/F | I | COL1A1 | E22 | c.1503delT | p.Ala502Leu/ fs*5 | SS (G/G) | Pp (A/C) |
| 5 | 5 | 57/M | I | COL1A1 | E22 | c.1503delT | p.Ala502Leu/ fs*5 | SS (G/G) | Pp (A/C) |
| 6 | 6 | 73/F | I | COL1A1 | E50 | c.3881A>T; 3882_3891del | p.Glu1294Val/ fs*32 | SS (G/G) | pp (C/C) |
| 7 | 7 | 137/M | III | COL1A1 | I51 | c.4248+1G>A/possible exon skipping | not applicable | SS (G/G) | PP (A/A) |
| 8 | 8 | 19/F | III | COL1A2 | E22 | c.1207G>T | p.Gly403Cys | SS (G/G) | Pp (A/C) |
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All of the mutations affected the coding sequences. In **COL1A1**, three mutations were single nucleotide deletions with subsequent frame shifts resulting in nonsense codons located 5, 32 or 76 nucleotides downstream of the deletion. Additionally, in one female patient the deletion was next to another missense mutation (73/F). In two patients, two mutations, both changing G>T/A, occurred in introns. In **COL1A2**, the mutation was a missense mutation changing glycine to cysteine in a female with type III OI.

The polymorphisms detected in collagen encoding genes

An analysis of sequencing chromatograms of the respective polymorphism sites in the genomic DNA revealed that all of the patients were homozygous for wild type Sp1 polymorphism in the **COL1A1** gene (Fig. 1 and Table 2). The PvuII polymorphism was detected in all three possible combinations (Fig. 1 and Table 2). Three females, two with type I and one with type III OI, were heterozygous for the PvuII polymorphism. Two males, one with type I and the other one with type III OI, were homozygous for the wild type (PP) genotype. The type I OI 73/F patient with a missense mutation followed by an immediate single base deletion and subsequent nonsense codon was homozygous for mutated allele of the PvuII polymorphism (pp). It is surprising that a single nucleotide deletion accompanied by homozygous “pp” polymorphism resulted in just a mild clinical phenotype.

**Effect of the mutations on secretion of procollagen type I**

The analysis of secreted procollagen type I from fibroblasts of three patients, two with OI type I and one with OI type III, revealed that in case of mutations in **COL1A1** no retention of procollagen alpha chains in the cells could be detected (right panel in Fig. 2A). However, alpha 2 chain could be detected by western blot analysis only as a fine band in precipitates from the culture medium obtained from fibroblasts of patients 91-D/F (OI type I) and 19/F (OI type III) (left panel in Fig. 2A). Procollagen alpha chains identified in the fibroblast culture medium of 73/F the patient (OI type I) migrated faster than the procollagen chains from control fibroblasts (lane marked 73/F in the left panel of WB blot in Fig. 2A). This resulted from a mutation in exon 50 of **COL1A1**, c.3881A>T. The mutation changed Glu at position 1294 to Val (Table 2). There was also a deletion of 9 nucleotides from position 3882 to 3891 (Table 2), which resulted in frame shift and a subsequent stop codon following the next 32 codons. In the culture medium collected from fibroblasts of the 19/F patient (OI type III) carrying a mutation in exon 22 of **COL1A2** (c.1207G>T) which changes one codon (Gly403Cys),
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only a very fine band corresponding to procollagen type I α2 chain was detected, whereas pro-α1 chain was represented by a much stronger band, indicating the ratio of α1:α2 to be higher than 2:1.

Although fibroblasts from the 91-D/F patient with a mutation in exon 2 (c.231delG) causing a frame shift (p.Thr78Pro), and a stop codon 76 codons downstream of the change, secreted the heterotrimeric procollagen type I, the pro-α2 chain was present in the culture medium at a lower amount than in the control (Fig. 2A).

An analysis of proteins in lysates of cultured fibroblasts following SDS/PAGE and WB did not reveal intracellular retention of pro-α1 chains in any of the analyzed fibroblasts. However, some non-specific cross-reaction of the anti-human type I procollagen antibodies was detected in lysates from all fibroblasts, including the control ones (Right panel in Fig. 2A). It is possible that the cross reaction of the antibodies was due to the presence of products of partial degradation of the type I procollagen chains. Poor detection of procollagen type I in the lysates of fibroblasts from patients 19/F and 91-D/F needs to be further investigated (Fig. 2A, right panel). In addition, problems with determination of thermal stability of procollagen type I in all three cases were probably due to the poor protein yields from the culture medium of the fibroblasts obtained from the OI patients (data not shown).

DISCUSSION

Mutation locations analyzed in this paper did not point directly to type of OI. The resulting changes in the encoded amino acids are not sufficient for predicting how severe the disorder will be. The consistency of the Glycine codon changes to the Cysteine codon (at position 403 in patient 19/F) correlates with commonly accepted
Gly to Xxx substitutions resulting in a more severe type III or a lethal type II of OI (Byers et al., 1991; Roughley et al., 2003). In addition, the possible exon skipping effect of I51 mutation at the 4248+1 position could explain the more severe type III phenotype manifested by a tremendous number of bone fractures (170 incidents within 26 years).

Presence of both “PP” and “SS” genotypes was linked to a two-fold lower risk of long bone fracture in children (Mann et al., 2001; Blades et al., 2010; Kurt-Sirin et al., 2014). The homozygous wild type status for both polymorphisms did lower the risk of bone fractures by half. Results reported by others revealed that the presence of at least one copy of the “s” allele of Sp1 polymorphism in COL1A1 gene increased the risk of long bone fractures prior to maturation by three-folds also in healthy children (Pace et al., 2008). No such correlation was found in dual energy X-ray absorptiometry results (DXA – means of measuring bone mineral density) among those children. Here, we did not find such a correlation probably because all of the patients were homozygous for wild type allele of the Sp1 polymorphism. The genotypic status for PromII polymorphism had no effect on bone fractures in patients with OI type III (Table 1).

Faster migration of procollagen alpha chains in patient 73 could be a result of weaker posttranslational modification, probably glycosylation, due to the C-propeptide of pro-α1 chain shortened 139 amino acids (Top alignment in Fig. 2B). In addition, it has been reported previously that correct pro-α(I) C-propeptide is critical for procollagen assembly (Suuriniemi et al., 2006). Therefore, one could speculate that the disturbance in the registry of the three procollagen chains due to different lengths of the C-propeptides from the three alpha chains, could lead to procollagen suicide. However, the non-mutated chains could be abnormally under-hydroxylated as an effect of insufficient amount of newly synthesized type I procollagen. Therefore, it could be processed faster through the ER and Golgi apparatus and more quickly secreted.

Disturbed ratio of procollagen chains in patient 19/F could be explained by shortage of the α2 chain, as α2 homo-trimer was secreted along with trace amounts of the hetero-trimer (Fig. 2A). The mutation is located in the region flanked by Gly-Xxx-Hyp triplets in both, α1 and α2 chains (Fig. 2C). This fragment also contains amino acids forming sites for interactions with interleukin 2, decorin and αβ integrin (Di Lullo et al., 2002) (Fig. 2C). Therefore, substitution of a small glycine residue by a larger cysteine in the pro-α2(Ⅱ) might disturb the structure of the triple-helix, decrease its stability and disturb binding sites for the above listed factors.

In case of patient 91-D/F it is possible that this extremely short polypeptide, containing just 152 amino acids due to a stop codon (bottom sequence in Fig. 2B), and mutated pro-α1 chain could be degraded, therefore, causing insufficient amount of procollagen type I to be secreted.

Results obtained by detection of mutations in genes encoding procollagen type I in patients diagnosed with either type I or III OI revealed that the causative mutations might occur anywhere in both genes. The clinical outcome resulting from these mutations is complex and the severity is difficult to predict based only on the type and location of the mutation, particularly when the mutation is dominant. Although recommendation for OI diagnosis based on a clinical exam and verified by DNA gene sequencing, but without protein analysis, is sufficient for clinical treatment, it seems to be insufficient for genetic counseling of both, the patient and the patient’s parents. Therefore, more research is needed on effects of pathogenic mutations on cell biology.

Conflict of interest
All authors declare no conflict of interests.

Acknowledgements
The authors like to acknowledge dr Antoni Pyrkosz, Anna Jakubiuk-Tomaszuk, Pawel Abramowicz, and Jerzy Konstantynowicz for constructive medical consultation of the manuscript.

Acknowledgements of financial support
The work was in part financially supported by Institutional grants KNW-1-010/P/1/0, KNW-1-007/P/2/0, and KNW-1-041/K/3/0 (all awarded to ALS). The equipment for molecular analyses used in this work was purchased using the EU funds (European Regional Development Fund) within the Sectoral Operational Program “Increase of Economic Competitiveness” (No.WK P1/14/3/2/2005/103/225/2007/1U) and Silesian Bio-Farma Center for Biotechnology, Bioengineering and Bioinformatics Project (no POIG.02.01.00-00-166/08 THE OPERATIONAL PROGRAMME INNOVATIVE ECONOMY FOR 2007-2013, Priority Axis 2). This study was also conducted with the use of equipment purchased by the Medical University of Białystok as part of the OP DEP 2007-2013, Priority Axis I.3, contract No. POPW.01.03.00-20-008/09.

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