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Osteogenesis Imperfecta: Ultrastructural and histological findings on examination of skin revealing novel insights into genotype-phenotype correlation

Running Title: Ultrastructural findings of skin in OI

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ABSTRACT

Background: Osteogenesis imperfecta (OI) is a heterogeneous group of inherited disorders of bone formation, resulting in low bone mass and an increased propensity to fracture. Over 90% of patients with OI have a mutation in COL1A1/COL1A2, which shows an autosomal dominant pattern of inheritance. In-depth phenotyping and in particular, studies involving manifestations in the skin connective tissue have not previously been undertaken in OI. Although protein analysis of cultured fibroblasts has historically been used in the diagnostic work-up of OI, other aspects of skin examination are not routinely performed as part of the diagnostic pathway in patients with OI.

Aims: To perform histological and ultrastructural examination of skin biopsies in a cohort of patients with OI; to identify common and distinguishing features in order to inform genotype-phenotype correlation and identify common and distinguishing features between the different subtypes of OI.

Methods: As part of the RUDY (Rare Diseases in Bone, Joints and/or Blood Vessels) study, in collaboration with the NIHR Rare Diseases Translational Research Collaboration, we undertook a national study of skin biopsies in patients with OI.

Results: We studied the manifestations in the skin connective tissue and undertook in-depth clinical and molecular phenotyping of 16 patients with OI. We recruited 16 patients: analyses has shown that in type 1 collagen mutation positive patients (COL1A1/ COL1A2) (n=4/16) consistent findings included: variable collagen fibril diameter (CFD), presence of collagen flowers. Histological examination in these 4 patients showed an increase in elastic fibres that are frequently fragmented and clumped.

Patients with phenotypic OI also appeared to have rare small collagen flowers on electron microscopy.

Conclusions: These observations provide evidence that collagen flowers and CFD variability are consistent features in OI due to type 1 collagen defects and reinforce the need for accurate phenotyping in conjunction with genomic analyses.

KEYWORDS: Osteogenesis Imperfecta, Electron microscopy, Type 1 collagen, COL1A1/COL1A2, collagen flowers, elastic fibres, collagen fibril diameter (CFD)

INTRODUCTION

Osteogenesis Imperfecta (OI) is a hereditary bone disorder commonly referred to as ‘Brittle Bone Disease’. Collagen is essential for the structural integrity of bones. Most people with OI either have a reduced amount of collagen, or abnormal collagen, due to mutations in the genes for type 1 collagen. This results in fragile, weak bones that have an increased risk of fractures. OI is primarily considered to be a Type 1 Collagenopathy (Gorlin RJ 2001) [1].

OI is categorised, according to Sillence, in 1979 into four main clinical phenotypes (Sillence et al., 1979) [2]. This classification was further revised in 2014 (Van Dijk and Sillence., 2014) [3]. Classical OI which includes types I-IV are mostly inherited in an autosomal dominant pattern, in which 90% of patients have a mutation in COL1A1/COL1A2 (Marini et al., 2007) [4]. Several other recessive forms of OI have been described more recently (Alanay et al., 2010; Dijk FS et al., 2009; Baldrige et al., 2008; Christiansen et al., 2010; Lapunzina et al., 2010; Martinez et al., 2012; Shaheen et al., 2012) [5-11].

We undertook a national study of in-depth phenotyping in OI with funding from the NIHR Rare Diseases Translational Research Collaboration (NIHR RD TRC). We studied manifestations in the skin connective tissue and undertook in-depth clinical and molecular phenotyping of patients with OI. We performed ultrastructural examination and phenotype-based assays of the skin connective tissue in patients with OI; to enable use of results in the diagnostic work-up of OI.

MATERIALS AND METHODS

The plan was to perform ultrastructural and histological examination of the skin connective tissue in OI to enable use of results in the diagnostic work-up. As part of a research project to study ultrastructure and histological findings of the skin connective tissue in OI, 16 patients

were recruited nationally to this study and skin biopsies undertaken. Genotyping was undertaken in all of the recruited patients and their clinical phenotype was studied in further detail. Ethical approval was obtained to undertake in-depth phenotyping and genetic work-up in this group of patients. Detailed history and clinical examination including a Dysmorphology assessment was performed in all the recruited patients. Further investigations including a skin biopsy (to perform histological and ultrastructural examination of the dermis) and detailed skeletal survey of patients with OI were performed.

Genomic DNA was extracted using standard protocols and targeted next generation sequencing performed for all the genes described in association with OI. The skin biopsy was performed after 1ml of 1% Lignocaine was injected on the inside of the right upper arm and a 3mm disposable biopsy punch used to obtain three samples for analysis. The specimens obtained from skin biopsy (n=16) were used for culturing fibroblasts, histology and electron microscopy.

For histological examination, the punch biopsy of skin (n=16) was fixed in buffered formalin, processed to paraffin wax, and two 4 μ m sections were prepared for staining with H&E or Orcein. The H&E stained section was assessed for normality of skin. The Orcein-stained section was assessed for elastic fibre area fraction and mean diameter of elastic fibres, presence of elastic fibre depletion, increase, or clumping. Elastic fibre area fraction in Orcein-stained sections was achieved by the point-counting method. Five areas of the dermis chosen randomly were analysed, including upper, mid and lower reticular dermis using a x40 objective and an eyepiece graticule. The mean fibre diameter was determined in the mid reticular dermis using the program NS-I Elements D3.0 (Nikon). The mean diameter and standard deviation of 50 fibres were measured in a captured image with a x20 objective, T-test was carried out with Graph Prism program. (Balasubramanian et al., 2015) [12].

Electron microscopy of the punch biopsy specimen (n=16) was performed by fixing it in 3% phosphate buffered glutaraldehyde, when fixed it was bisected or trisected longitudinally. The tissue was then post-fixed in 1% osmium tetroxide, processed to epoxy resin and at embedding orientated to display sections of epidermis and full thickness dermis. The dermis was assessed and thin sections were cut and examined using a Philips 400 TEM, image capture and collagen fibril diameter quantitations were performed using an AMT 16 megapixel mid mount digital camera. Collagen fibrils were examined for the presence, and relative frequency, of collagen flowers were analysed. Upto 18 grid meshes/ patient were morphometrically examined. The diameter of 30 - 60 unselected collagen fibrils, sectioned transversely, within collagen bundles away from adnexal structures, was measured and note was made of the mean diameter, the range, and the deviation root mean squared (RMS) at the papillary, upper and mid-reticular dermis in three different bundles, and deep reticular dermis. The mean of the means in the reticular dermis was calculated. The diameter of approximately 20 collagen bundles, in the mid reticular dermis was measured, at two different magnifications (x120 and x550).

Although, 16 patients were recruited to the study, in this paper, we present in-depth molecular and phenotyping data of 4 patients with a type 1 collagen (COL1A1/COL1A2) mutation. The remainder patients do have an established genetic aetiology yet and whole exome sequencing (WES) studies are ongoing and their results will be reported separately.

CLINICAL SYNOPSIS & RESULTS

Below is a brief clinical description and results from the skin biopsy analyses in patients with type 1 collagen mutations (n=4):

Patient 1: Four-year-old boy, the first child to healthy, non-consanguineous, Caucasian parents with no significant family history. The pregnancy was uncomplicated and he was born by elective caesarean section at term, due to a breech presentation, with a birth-weight of 3.1kg (9th-25th centile). In the first year of life, there were concerns with poor feeding and weight gain. He was noted to have significant joint laxity, delayed gross motor skills, blue sclerae and had surgery for bilateral undescended testes. He sustained fractures of his ribs, femur and skull and had been commenced on treatment with Pamidronate. His weight and height were below the 0.4th centile, with preservation of head circumference. On examination, he had relative macrocephaly with a triangular face, broad forehead, beaked nose and significant joint laxity. Skeletal survey showed Wormian bones, multiple vertebral compression fractures, flared metaphyses and dense metaphyseal lines consistent with bisphosphonate therapy and normal appearance to the ribs, consistent with Type IV OI. Genetic testing identified a glycine substitution in COL1A1, c.599G>T,p.Gly200Val in exon 8, consistent with a diagnosis of type IV OI (cDNA ref. sequence NM 000088.3).

Results: Histology showed no abnormality on H&E staining. Elastic fibres appeared increased mainly in the upper reticular dermis. Area fraction and mean fibre diameter are shown on Table 1 (Figure 1.1). Electron microscopy showed novel findings including, fibroblasts with mildly-dilated protein-filled rough endoplasmic reticulum and fibroblasts secreting non-fibrillary collagen. Macrophages throughout the dermis present phagocytosing non-fibrillary collagen (Figure 2.1). Mean CFD was significantly smaller than expected for age (Balasubramanian et al., 2015) [12]. Moderate numbers of collagen fibril profiles were irregular in outline with occasional, rare small collagen flowers. CFD variability was not excessive with moderate amount of non-fibrillary collagen present in inter-bundle space. Collagen bundle diameter and packaging were unremarkable.

Patient 2: 9-year-old boy, the second child to healthy, non-consanguineous, Caucasian parents with no significant family history. The pregnancy was uncomplicated and he was born at term with a birth-weight of 4.05kg (75th-91st centile). At eleven months of age, he sustained a fracture of his clavicle, but remained well thereafter. He had normal development. He went on to develop significant joint laxity and had two fractures involving his tibia and elbow. He currently attends a mainstream school.

On examination, he has greyish-blue sclerae, flat forehead, high-arched palate, 'S' shaped spine, hypermobility and flat feet. Growth parameters included: height ~ 160cms (99.6th centile), weight ~ 53kg (91st-98th centile) and head circumference ~ 59.5cms (98th-99.6th centile), with no evidence of asymmetry. Skeletal survey showed 'S'- shaped scoliosis and healed clavicle fracture. Genetic testing identified a mutation in COL1A1, c.3421C>T in exon 47 (cDNA ref. sequence NM 000088.3), which is predicted to replace the arginine at position 1141 of the triple helix domain with a premature termination codon and lead to a truncated protein, which is consistent with a diagnosis of type I OI.

Results: Skin biopsy showed increased area fraction of elastic fibres with clumping on orcein-stained sections. (Figure 1.2). Collagen fibres in the dermis appeared thin and interspersed with myxoid material, appearances consistent with a collagen abnormality. Multiple small, medium and large sized-collagen flowers (with an average diameter of 488 nm and the largest collagen flower diameter of 721 nm) were noted on electron microscopy. Mean collagen diameter was 61 nm (normal for age~ 70 nm), therefore less than expected for age (Figure 2.2) (Balasubramanian et al., 2015) [12].

Patient 3: 16-year old boy, first child of healthy, non-consanguineous Caucasian parents with no significant family history. The pregnancy was uncomplicated and he was born at term with a birth weight of 3060 grams and was in a good condition immediately after birth. He sustained his first fracture at a month of age and went to sustain several fractures needing treatment with Pamidronate. He currently attends a mainstream school. His weight and

height were below the 0.4th centile, with preservation of head circumference. On examination, he had blue sclerae, joint laxity and not dysmorphic. Skeletal survey confirmed the presence of multiple Wormian bones (abnormal intra-sutural bones that are a radiological feature in OI), osteopenia and multiple healed fractures. Genetic testing identified a heterozygous pathogenic frameshift mutation c.2010delT in exon 30 of COL1A1(cDNA ref. sequence NM 000088.3), which is predicted to result in a premature termination codon 94 amino acids downstream, consistent with a diagnosis of OI.

Results: Histological examination revealed increased elastic fibre area fraction with increase in mean thickness (Figure 1.3). Electron microscopy revealed numerous collagen flowers (with an average collagen flower diameter of 342 nm and the largest collagen flower diameter of 626 nm) in moderate numbers of collagen bundles, mean CFD of 73nm (lesser than expected for age- normal~85nm) (Balasubramanian et al., 2015) [12] and mean collagen bundle diameter of 4.1µm. The most striking finding was the presence of multiple large collagen flowers which are distinctive and several in the same plane (Figure 2.3). The collagen flowers appeared to be made up as a composite of a number of adjacent collagen fibrils with no spiral collagen fibrils being seen. **Patient 4:** 61-year old patient of Canadian origin who presented with an extensive fracture history and significant joint laxity. She was born with a left-sided congenital dislocation of the hip which was treated surgically at two years of age. She did not have any evidence of abnormal scarring and no evidence of skin hyper-extensibility. On examination, she had greyish-blue sclerae, not dysmorphic and a high-pitched voice. Genetic testing identified a heterozygous c.81delA mutation in exon 1 of COL1A1(cDNA ref. sequence NM 000088.3). This mutation is predicted to result in a frameshift at p.Val28 and a truncating stop codon 45 amino acids downstream, consistent with a diagnosis of OI.

Results: Histological examination showed prominent elastic fibres on light microscopy. Orcein-stained sections revealed increased elastic fibre area fraction, which were frequently

clumped (Figure 1.4). Electron microscopy revealed moderate numbers of large collagen flowers in mid and deep reticular dermis (Figure 2.4), mean CFD of 66nm (lesser than expected for age and mean collagen flower diameter of 336 nm (Balasubramanian et al., 2015) [12].

The results from the skin biopsy (histology and EM) in all patients is summarised in Table 1.

DISCUSSION

OI is variable in severity and can be associated with normal stature and very few fractures to being perinatally lethal. The patients described in this paper (Patients 1-4) have a bone fragility phenotype with joint laxity and a significant fracture history with a molecularly confirmed type I collagen defect (Database of type 1 collagen mutations) [13].

In terms of the specific COL1A1/A2 mutations identified in this cohort, this has provided us with insight into the pathogenesis and the effect at the tissue/protein level and how this would manifest in the skin connective tissue. Histological examination of skin biopsy in patients examined within this cohort has shown abnormal elastic fibre area fraction. Clumping and fragmentation of elastic fibres appears to be a consistent finding in patients with COL1A1 mutations. The proportional increase in the amount of elastic fibres reflects the abnormalities in the collagen rather than in the elastic fibres.

Electron microscopy in all the mutation positive patients examined has consistently shown increased variation in collagen fibril diameter (CFD), variation from expected mean CFD and the presence of collagen flowers. In neonatal skin, the mean reticular dermal collagen fibril diameter (CFD) is 70nm; in maturity at 20 years of age; it rises to 90nm and in old age, drops back to 70nm. Collagen fibrils are long, thin and in cross-section are round. In longitudinal section, the collagen fibrils are banded at a periodicity of 60 - 64nm, and are surrounded by a

wire-net of collagen VI. The bundles of collagen fibrils are bounded by spindle-shaped fibroblasts.

Fibroblasts actively secreting fibrous collagen contain deep invaginations, which contain a few thin collagen fibrils known as fibripositors. These collagen fibrils grow laterally away from the fibroblast. Fibroblasts also secrete the component parts of elastic fibres. Elastic fibres are composed of microfibril around which is deposited hyaline elastin. The amount of elastin present in the elastic fibre increases in reticular dermis relative to papillary dermis. In normal skin, the volume fraction of elastic tissue decreases from newborn up to maturity, there after it increases again due to an increase, followed by a decrease in collagen synthesis. A proportion of fibroblasts wraps tightly around elastic fibres and actively secretes matrix metalloproteinases. These fibroblasts contain enlarged lysosomes. There are occasional macrophages and mast cells that also secrete proteinases. In between the collagen bundles there are proteoglycan molecules with attached glycosaminoglycan particles. These are in greater amounts in the papillary dermis relative to the reticular dermis. Fibroblasts are attached to numerous microfibrils at regular attachment plaques. Interestingly, Patient 1 had macrophages identified throughout the dermis with presence of phagocytosing non-fibrillary collagen and moderate numbers of collagen fibrils were identified with an irregular profile. This appearance on ultra-structural examination of the skin is a novel finding in OI.

Collagen fibrils can exceed twice their normal diameter and appear in transverse section as flower-like. These abnormal collagen fibrils in longitudinal section appear to be fraying or splitting and, by scanning electron microscopy, appear to have spiral grooves. These are often described as ‘collagen flowers’ and are usually seen in patients with Classical Ehlers-Danlos syndrome (EDS), associated with mutations in the COL5A1/A2 gene. Collagen flowers can vary in diameter from 100 – 300nm. In this condition, collagen flowers can be found within well-formed collagen bundles and within areas of loose connective tissue between collagen bundles. They are found at any point within the reticular dermis. Moderate

numbers of collagen flowers are also seen in a range of skin pathologies such as: Actinic damage, inflammation, Amyloid, Lymphoma and Pseudoxanthoma Elasticum (PXE), thus underscoring the importance of full knowledge of clinical details and site of skin biopsy before suggesting a diagnosis. Collagen flowers can vary in diameter from 100 – 300nm. In Classical EDS, the frequency and size of collagen flowers shows some correlation with severity of symptoms, where the more flowers, the greater the severity (Hausser & Anton-Lamprecht., 1994; Holbrook & Byers., 1982; Ghadially F., 1997) [14-16].

There is minimal literature on large scale ultrastructural examination of dermis in OI. This study provides morphological insight into the effect of gene mutations at the tissue level. Patients with a type 1/IV OI appear to have variability in CFD and collagen flowers as a consistent finding whilst patients without a type 1 collagen defect do not have the same findings on skin biopsy. Hence, we have been able to gain a better understanding of the molecular pathogenesis in OI, which would help provide further insights into the disease, particularly with the increasing use of genomic sequencing studies which are likely to reveal several variants of unknown significance.

Specific changes have been identified in protein analysis and ultrastructural examination of the dermis in type 1 collagen mutation-positive patients. These changes segregate with mutations in the type 1 collagen genes. The presence of alterations in the skin morphology in other recruited patients also supports the idea that there are significant changes at the ultrastructural level in the patients studied. Therefore, the skin findings provide us with better discrimination of the different phenotypes and insight in to the possible mechanism for these changes in the skin biopsy. Even in the era of next generation sequencing and targeted gene panel testing in OI, histological and ultrastructural examination of the skin will still play a role as an adjunct investigation in trying to distinguish patients and determine significance of known and novel gene variants identified through whole exome/ genome sequencing.

FIGURE LEGENDS

Figure 1: Punch skin biopsy of Patients 1-4 in this study. Morphometry of elastic fibres stained with Orcein showed increased area fraction of elastic fibres when compared to expected for age group. (Orcein, original magnification 40x).

Figure 2: Electron microscopy images of Patients 1-4 in this study. Arrows indicate collagen flowers (Original magnification of 20,000x).

Patient 1a and b: EM showing mis-shaped collagen fibrils in reticular dermis with a mean CFD of 55nm which is below expected for age and upper reticular dermis with presumed macrophage phagocytosing un-polymerised collagen.

Patient 2: EM showing multiple large collagen flowers in the mid-reticular dermis with lower mean CFD than expected for age.

Patient 3: EM showing numerous collagen flowers in moderate numbers of collagen bundles with lower mean CFD than expected for age.

Patient 4: EM showing numerous large collagen flowers in mid and deep reticular dermis.

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Translational Research Collaboration. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

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Table 1: Elastic fibre area fraction, mean elastic fibre thickness and EM data with genetic results in patients with OI (1-4)

Patient	Age at Bx (months)	Elastic Fibre Area Fraction (95% CI)	Elastic Fibre mean thickness±SD in µm (95% CI)	Mean CFD±RMS in nm (range)	CFD relative to age (Estimate in brackets)	Collagen Flowers Incidence	Irregular profile	Molecular genetics
1	(46) 3yrs 10 m	0.22 (0.13-0.17)	3.2±0.94 (4.23-4.9)	55±6.2 (21 – 70)	55 (72-75) Less than	No	Yes	COL1A1 .c.[599G>T];[=],p.[(Gly200Val)];[=] Exon 8
2	(110) 9yr 2 m	0.21 (0.13-0.17)	4.43±2.29 (3.86-4.62)	61±6.1 (45-81)	61 (75-80) Less than	Moderate	Yes	COL1A1 .c.[3421C>T];[=] in exon 47
3	(178) 14yr 9m	0.10 (0.07-0.9)	4.95±1.67 (3.86-4.62)	73±6.8 (54-116)	73 (82-86) Less than	Numerous	No	COL1A1,c.2010delT,p.[(Gly671fs)];[=]
4	(674) 56yr 1m	0.18 (0.09-0.15)	4.91±1.29 (4.25-5.0)	66±6.4 (48-89)	66 (75) Less than	Moderate	No	COL1A1,c.81delA,p.[=]+[Val28fs]

Bx: Biopsy; CI: Confidence Interval; SD: Standard Deviation; CFD: Collagen Fibril Diameter; RMS: Root Mean Square