ventional mutation scanning methods may miss submicroscopic deletions.

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Homozygosity for a splice site mutation of the *COL1A2* gene yields a non-functional pro α 2(I) chain and an EDS/OI clinical phenotype

EDITOR—Type I collagen is the major structural protein of skin, bone, tendon, ligaments, and cornea. It is a heterotrimer of two α 1(I) chains and one α 2(I) chain. Mutations in one of its two structural genes (*COL1A1*, *COL1A2*) underlie the inherited disorders osteogenesis imperfecta (OI) and Ehlers-Danlos syndrome types VIIA and B (EDS VIIA, EDS VIIB).¹ Mutations which inhibit the processing of the protein precursor, procollagen, cause the

ligamentous laxity and extreme joint hypermobility of EDS VII. Structural abnormalities and/or reduced production of type I collagen lead to the increased bone fragility, thin skin, blue sclerae, dentinogenesis imperfecta, and presenile deafness of osteogenesis imperfecta. The OI phenotype can vary from perinatally lethal to mild disease depending upon the nature of the mutation. Among the mild group are patients who are heterozygous for a non-functional *COL1A1* allele.²⁻⁵ Homozygosity for a non-functional *COL1A1* allele appears to be incompatible with life.⁶ Several years ago we described a male infant who was totally deficient in α 2(I) chains owing to homozygosity for a non-functional *COL1A2* allele.⁷⁻⁹ He had the severe, progressively deforming type of osteogenesis imperfecta (OI type III). His heterozygous third cousin parents, however, showed no overt clinical symptoms but

Figure 1 Pedigree of family.

were considered to be prematurely osteoporotic following *x* ray examination. Subsequently, two further patients lacking $\text{pro}\alpha 2(\text{I})$ chains from type I collagen were reported.^{10,11} However, their clinical phenotypes lacked any of the skeletal abnormalities of osteogenesis imperfecta but included generalised joint hypermobility, skin hyperextensibility, and scarring typical of the Ehlers-Danlos syndrome. We have now identified another patient homozygous for a mutation yielding non-functional *COL1A2* alleles whose clinical phenotype showed generalised joint hypermobility and foot deformities reminiscent of Ehlers-Danlos syndrome associated with pale blue sclerae and a mild increase in bone fragility characteristic of osteogenesis imperfecta.

The proband is the first child of first cousin parents (fig 1). Marked ligamentous laxity and muscle hypotonia had been first noted at her premature (28 weeks' gestation) birth. When ambulation was significantly delayed she was investigated for muscular dystrophy but, following a normal muscle biopsy, the motor delay was attributed to her ligamentous laxity. On clinical examination at 9 years of age she was of average height but with marked generalised joint laxity, pes planus, and valgus heels leading to a secondary shortening of the achilles tendon. Her skin was normal, her sclerae were pale blue, and there was dental overcrowding but no dentinogenesis imperfecta. The limbs were straight and normal both on examination and by *x* ray. There was a history of recurrent patellar dislocations and fractures of the skull, clavicle, fingers (three), and a toe following separate minimal traumas. An asymptomatic early systolic murmur present on initial presentation had spontaneously resolved. Her cardiovascular system, ECG, and echocardiography were normal. Her mother showed some joint laxity, but her father was not available for examination. She has two younger sisters who have both progressed normally. A paternal cousin was reported to have joint hypermobility and fractures following minimal injury.

Skin fibroblast cultures were established from both the proband and her mother. The cells were labelled with ¹⁴C-proline (1 $\mu\text{Ci}/\text{ml}$).¹² Proteins were harvested from the medium by ethanol precipitation and from the cells by lysis with 0.5 mol/l acetic acid/0.5% triton X-100 at 4°C. Medium and cell layer procollagens were converted to collagens by limited pepsin digestion of the native protein.¹² Procollagen and collagen chains were analysed on 5% polyacrylamide gels containing 2 mol/l urea using the tris-glycine-SDS buffer system.¹³ Procollagen samples were reduced with 1% mercaptoethanol before electrophoresis. The SDS-polyacrylamide gels of the procollagens secreted by the proband's cells showed

Figure 2 SDS-polyacrylamide gels of medium procollagens and pepsinised collagens from medium and cell layer of patient (P) and control (C) fibroblasts.

$\text{pro}\alpha 1(\text{I})$ and $\alpha 1(\text{I})$ bands but no evidence of $\text{pro}\alpha 2(\text{I})$, $\text{pNa}2(\text{I})$, or $\alpha 2(\text{I})$ bands (fig 2). After limited pepsin digestion the collagens from both medium and cell layer of the proband's cells showed only $\alpha 1(\text{I})$ chains. These migrated more slowly on SDS-PAGE than those from a control cell line (fig 2) indicative of increased post-translational modification. The chains of other collagens, for example, type III and type V, from the patient's cells were not affected.

Cytoplasmic RNA, isolated from fibroblasts by the NP 40 lysis technique,¹⁴ was reverse transcribed and PCR amplified to yield several overlapping fragments encoding the whole $\alpha 2(\text{I})$ chain. Products were analysed on agarose or polyacrylamide gels. The proband's RNA gave a product for each primer pair; however one product was slightly smaller than the corresponding fragment from the control RNA (fig 3A). Although this smaller fragment was the predominant product from the proband's RNA, two minor higher molecular weight bands visible in polyacrylamide gels suggested heteroduplex formation and implied the existence of a second molecular species in the RT-PCR reaction. This fragment pattern was consistent in replicate reverse transcriptions of duplicate RNA preparations from the proband (fig 3A). The proband's abnormal product was inserted into M13 vectors and the sequences of multiple clones compared to that of the wild type fragment from control RNA. All of the proband's clones examined had an identical mutant sequence which, compared to the normal control, was missing the last 17 bp of *COL1A2* exon 46 (fig 3B). The resulting frameshift introduces a premature termination codon after three residues. To characterise the minor components in the proband's RT-PCR reaction, both higher molecular weight bands were excised from a polyacrylamide gel, eluted, and reamplified. Each gave two bands of approximately equal intensity on ethidium bromide stained polyacrylamide gels; one band corresponded to the deleted product, the other was the size of the normal fragment (fig 3C). Several M13 clones of each product were sequenced and yielded two distinct sequences, one showing the 17 bp deletion and the other the normal $\alpha 2(\text{I})$ mRNA sequence.

To assess the relative abundance of the different $\alpha 2(\text{I})$ mRNAs, equal amounts of the mother's, proband's, and control RNAs were RT-PCR amplified for only a limited number of cycles with one of the primers ³³P end labelled. The products were analysed by polyacrylamide gel and autoradiography. In the mother, the normal sized product was predominant with the deleted product representing only a very minor component. The patient gave a similarly weak signal for the deleted product (fig 3D).

Figure 3 (A) 5% polyacrylamide gel of RT-PCR products from the proband and three control RNAs. Lanes marked P₁ are duplicate RTs of the patient's first RNA preparation, lane marked P₂ is an RT from a second RNA preparation from the patient, C₁, C₂, and C₃ are RTs from control RNAs, L is a 1 kb ladder size marker (Gibco-BRL). (B) Sequencing gel of the antisense clones of cDNA from patient and control RNAs. (C) 5% polyacrylamide gel of PCR reamplifications of gel isolated fragments and heteroduplexes showing control RT (C), upper heteroduplex (U), lower heteroduplex (L), and patient's major component (P). M is the 1 kb ladder size marker. (D) Autoradiograph of 5% polyacrylamide gel of RT-PCR reactions using equivalent amounts of RNA and radioactively labelled primer. (P) proband, (M) her mother, and (C) a control.

Genomic DNA from the proband, her mother, and a control were PCR amplified using primers located upstream of the deletion in exon 46 and downstream in exon 47 of *COL1A2*. Each DNA yielded single products of equal size. These were ligated into the M13 derivatives BM20, BM21 (Boehringer-Mannheim) for sequencing. Every clone from the proband had a C instead of T as the second base of IVS 46 (fig 4A). Clones of the mother's DNA showed two changes from the control. Half her clones showed the C for T⁺ substitution seen in the proband, the others had the normal IVS46 T⁺ sequence but had an A instead of a G at position +42 of IVS 46. The T⁺→C⁺ mutation creates a new restriction site for the enzymes *BbvI* [GCAGC(N)₃] and *TseI* [GC(A/T)GC]. These enzymes were used to digest amplified genomic

DNA from the proband's blood and fibroblasts and blood from her mother, her two sisters, and four unrelated controls. The digests were electrophoresed on polyacrylamide gels (fig 4B). Both enzymes confirmed that the proband was homozygous for the mutation and the mother was a heterozygous carrier. The older of the proband's two sisters was also shown to be a carrier of the mutant allele but the second sister had two normal alleles.

The mutation that we have identified in this proband substitutes the obligate T⁺ of the donor splice site -cGTAAGT- of *COL1A2* IVS46 with a C. When such mutations arise in collagen genes, the usual outcome is skipping of the preceding exon from the mRNA to yield a shortened protein which retains its reading frame and the collagen triplet (Gly-X-Y) amino acid sequence. However,

Figure 4 (A) Sequencing gels of cloned genomic DNA fragments from a control, the proband, and both alleles of the proband's mother. The base change T⁺→C⁺ is marked by the larger arrows, the G⁺→A⁺ (IVS46) is marked with smaller arrows. (B) 5% polyacrylamide gels of *BbvI* and *TseI* restriction enzyme digests of amplified genomic DNA fragments from the proband, her mother, her sisters, and four controls C₁-C₄. Lane U is undigested product, L is the 1 kb size marker.

in this case there was no evidence for an exon skipped product; instead the predominant product arose from use of a cryptic donor splice site using the second and third bases of a glycine codon 17 bp upstream of the normal splice junction as the obligate -GT- dinucleotide. Calculations according to the Shapiro and Senapathy rules¹⁵ yield a score of 79 for the normal and 60.8 for the mutant splice site sequences. The cryptic site generates a score of 72.8. There is a potential -GT- sequence closer to the original splice site which yields a score of 62 but this is not used. Thus, for most of the mature mRNA the last 17 bp of exon 46 are deleted and the resultant frameshift introduces a new termination codon just three codons further downstream. The weak signal from the proband and the lower yield of the deleted product from the heterozygous mother obtained by semi-quantitative RT-PCR of equal amounts of RNA indicate that mutant messenger RNA is less abundant than normal. Messenger RNAs containing premature termination codons are frequently destroyed by a process known as nonsense mediated decay.¹⁶ If any of the mutant message was translated, the pro $\alpha 2(I)$ chains would lack a C propeptide domain and thus would not be incorporated into triple helical molecules. As a consequence, the proband produces a type I collagen homotrimer molecule containing three $\alpha 1(I)$ chains instead of the normal heterotrimer with two $\alpha 1(I)$ and one $\alpha 2(I)$ chains. The $\alpha 1(I)$ homotrimer triple helices form less readily than heterotrimer molecules and are thus subjected to increased post-translational modification leading to slow migration of the chains on SDS-PAGE gels. Mutation of the obligate -GT- of a donor splice site should totally abolish splicing at that junction; however, in this case the faint heteroduplexes observed in the proband's RT-PCR contained some normal product, suggesting that a limited amount of normal splicing had occurred at the mutated site despite it having a lower score than alternative cryptic sites in the vicinity. Any protein produced from these normal transcripts was undetectable in the cell culture analyses.

Patients with a total $\alpha 2(I)$ deficiency are extremely rare, this latest patient is only the fourth to be described, and they show markedly different clinical phenotypes. In the original case, homozygosity for a 4 bp deletion within the $\alpha 2(I)$ C propeptide coding region meant that the pro $\alpha 2(I)$ chains synthesised could not be incorporated into type I collagen molecules and $\alpha 1(I)$ trimers were formed.⁸ The patient had an extremely severe, progressively deforming osteogenesis imperfecta with multiple fractures, severe skeletal deformities, and popcorning of the epiphyses.⁷ His heterozygous third cousin parents who made half normal amounts of pro $\alpha 2(I)$ chains suffered from mild joint laxity but appeared clinically normal until x ray examination indicated premature osteoporosis. Two later accounts of Japanese patients^{10,11} reported an absence of $\alpha 2(I)$ chains in skin fibroblast cultures from patients who presented with joint hypermobility, hyperextensible skin, and scarring characteristic of the Ehlers-Danlos syndrome. Aortic regurgitation was also observed but no skeletal abnormalities were recorded. In the first of these cases the parents were second cousins suggesting possible homozygosity¹⁰; in the second the parents were unrelated and compound heterozygosity was postulated.¹¹ Although a reduction in $\alpha 2(I)$ mRNA was reported in one of these patients,¹¹ no gene defects were defined. Why apparently similar protein abnormalities should produce such diverse clinical phenotypes is unclear. One could speculate that the unknown

mutations in the Japanese patients affected a tissue specific gene promoter or enhancer limiting the expression of the defect to soft tissues and sparing the skeleton. However, in the other two cases the $\alpha 2(I)$ defects result from structural abnormalities within the *COL1A2* gene and should be expressed wherever the gene is active. As such they might be expected to generate similar clinical phenotypes but our latest proband is much more mildly affected than the original patient and shows none of his skeletal deformity or severe osseous fragility. This could be the result of epigenetic factors or perhaps the minute amount of normal $\alpha 2(I)$ detected as heteroduplexes in the RT-PCR reactions, although not as protein in cell cultures, is sufficient to ameliorate the clinical phenotype from a severe OI to a much milder EDS/OI.

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Mutation and haplotype analysis of the *CFTR* gene in atypically mild cystic fibrosis patients from Northern Ireland

EDITOR—Although cystic fibrosis (CF) is a monogenic disorder of autosomal recessive inheritance, it displays a diverse clinical phenotype. Over 1000 molecular lesions in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene together with the action of modifying genes can result in the variable expression of CF.^{1,2}

Classical CF causes dysfunction of the lung, sweat glands, testis, ovary, intestine, and pancreas.³ However, there is considerable variation in measurements of the onset age, presence of pancreatic insufficiency, sweat electrolyte levels, and progression of lung disease. Particularly mild manifestations of cystic fibrosis are conveniently grouped as "atypical CF" and result from a different *CFTR* mutation spectrum from classical CF patients.⁴⁻⁸ Recognition of the wide range of disease presentation in CF is important for appropriate treatment and effective counselling of those at risk. The repertoire of other disorders associated with *CFTR* variants include various respiratory afflictions such as asthma,⁹ chronic bronchitis, disseminated bronchiectasis,^{6,7,10} allergic bronchopulmonary aspergillosis,¹¹ nasal polyposis,¹² chronic pancreatitis,¹³ and certain forms of male infertility characterised as congenital absence of the vas deferens (CAVD) and obstructive azoospermia.^{8,14}

This report details the *CFTR* variants, characterised by fluorescent sequencing after screening the entire coding and surrounding intronic sequences by denaturing gradient gel electrophoresis (DGGE), in a panel of 31

unrelated atypical CF patients from Northern Ireland. The frequencies of these variants in the normal population was also examined. Automated detection of fluorescently labelled multiplex PCR fragments was used to type three intragenic *CFTR* dinucleotide repeats for assigning micro-satellite haplotypes to the atypical CF alleles.¹⁵ The genotypes for a functionally important polythymidine branch/acceptor site of intron 8 in the *CFTR* gene (Tn=IVS8-6(T)n)¹⁶ were also typed for our CF, atypical CF, and normal chromosomes.

The affected subjects from Northern Ireland were diagnosed during neonatal CF screening by two increased levels of immunoreactive trypsin from Guthrie card samples, followed by two sweat tests at one week intervals.¹⁷ Older patients (those currently over 14 years) were diagnosed by sweat testing after referral on clinical suspicion. Diagnosis of "classical" CF is generally based on a pathological sweat test, a *CFTR* mutation on each CF allele, and typical clinical symptoms of gastrointestinal and pulmonary disease.³ Diagnostic problems arise when one or more of the above criteria are not fulfilled. For example, Highsmith *et al*¹⁸ reported CF gene mutations in patients with normal sweat chloride concentrations. The highest sweat electrolyte results for the atypical CF group were in the diagnostic range for CF (table 1), although they tended to provide borderline results which required repeating before diagnosis was made. The overall clinical course is much milder than for our CF patients, as shown by slower decline on chest x ray appearances, Shwachman scores, deviation of height and weight below the mean, and decline in pulmonary function. They did not present with meconium ileus and only a few have had *Pseudomonas aeruginosa* infections (table 1). Only about half of the patients receive pancreatic

Table 1 The *CFTR* genotypes and clinical details for a panel of 31 atypical CF patients from Northern Ireland

Patient	<i>CFTR</i> mutations	Tn genotype*	Sex/age (y)	Sweat chloride†	Bacterial infection‡	Pancreatic status§	Shwachman score¶	FEV ₁ %¶
1	ΔF508/R75Q + 5T	9/(11) 5	M/32	93	PA	PI	18/25	80
2	ΔF508/R117H + 5T	9/(12) 5	M/9	74	HI	PI	21/25	90
3	ΔF508/R75Q	9/7	F/32	65	n	PS	23/25	92
4	ΔF508/5T	9/(11) 5	M/7	83	n	PS	24/25	—
5	ΔF508/U	9/7	F/23	81	HI	PS	19/25	87
6	ΔF508/U	9/7	F/11	63	n	PS	23/25	103
7	ΔF508/U	9/7	F/24	103	PA	PI	13/25	66
8	ΔF508/U	9/7	F/13	83	n	PS	18/25	81
9	R75Q/U	7/7	F/20	117	HI	PI	20/25	88
10	R75Q/U	7/7	M/6	84	HI	PS	19/25	—
11	R75Q/U	7/7	M/33	96	n	PI	21/25	86
12	S1235R/U	x/x	F/18	69	HI	PI	24/25	90
13	S1235R/U	7 or 9/7 or 9	M/22	76	n	PI	20/25	119
14	R117H + 5T/U	(12) 5/7	M/17	91	PA	PI	20/25	77
15	R297Q + 5T/U	(11) 5/7	F/14	62	n	PI	—	—
16	5T/U	(11) 5/7	M/13	79	n	PI	21/25	75
17	5T/U	(12) 5/7	F/20	74	n	PS	22/25	100
18	5T/U	(11) 5/7	M/28	55	n	PI	19/25	68
19	U/U	7/7	M/8	58	HI	PS	19/25	94
20	U/U	7/9	M/26	74	n	PS	23/25	106
21	U/U	9/7	M/8	55	HI	PS	22/25	—
22	U/U	7/7	M/8	77	n	PS	21/25	—
23	U/U	7/9	M/12	58	HI	PI	21/25	92
24	U/U	7/7	M/12	61	n	PI	19/25	91
25	U/U	7/7	M/21	72	n	PS	22/25	96
26	U/U	7/7	F/7	89	n	PI	21/25	87
27	U/U	7/7	M/8	65	n	PS	23/25	—
28	U/U	7/7	F/15	78	HI	PI	20/25	76
29	U/U	7/7	F/28	111	PA	PI	16/25	72
30	U/U	9/7	M/10	84	n	PS	20/25	81
31	U/U	9/7	M/9	66	n	PI	22/25	93

*The number of TG repeats (IVS8GT) upstream of the Tn tract is given in brackets only before a T5 score as their number can modulate alternative splicing only when this is activated by the T5 allele.¹⁶ U = unknown (no mutation detected), M = male, F = female, x = uncharacterised.

†High sweat levels, diagnostic of CF, are those above 70 mmol/l chloride, borderline levels are 40–70 mmol/l, and normal levels are below 40 mmol/l.¹⁷ In the atypical cohort, 20 patients have high sweat levels and 11 have borderline values.

‡HI = *Haemophilus influenza*, PA = *Pseudomonas aeruginosa*, n = no bacterial growth detected.

§PI = pancreatic insufficient, PS = pancreatic sufficient.

¶FEV₁/FVC % = forced expiratory volume in one second / forced vital capacity, as a percentage. — = no value available.