ELECTRONIC LETTER

Mutation of *COL11A2* causes autosomal recessive non-syndromic hearing loss at the DFNB53 locus

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Background: Allele variants of *COL11A2*, encoding collagen type XI α2, cause autosomal dominant non-syndromic hearing loss (ARNSHL) at the DFNA13 locus (MIM 601868) and various syndromes that include a deafness phenotype.
Objective: To describe a genome-wide scan carried out on a consanguineous Iranian family segregating ARNSHL.
Results: Genotyping data identified a novel locus for ARNSHL on chromosome 6p21.3, which was designated DFNB53. Homozygosity for the P621T mutation of *COL11A2* was present in all deaf persons in this family; this same variation was absent in 269 Iranian controls. Sequence comparison of collagen type XI α1 and α2 peptides across species shows that the replaced proline is an evolutionarily conserved amino acid.
Conclusions: The P621T mutation of *COL11A2* affects the Y position of the canonical -Gly-X-Y- repeat in collagens. It lies near the amino-terminus of the triple helical region and causes ARNSHL. This finding suggests that mutation type and location are critical determinants in defining the phenotype of *COL11A2* associated diseases.

Hearing loss is the most common sensory impairment. It affects one of every 500 newborn infants,[1][2] and another one of every 1000 children becomes severely or profoundly hearing impaired before adulthood.[3] In most cases, additional phenotypically distinguishing signs and symptoms are absent, consistent with the diagnosis of non-syndromic hearing loss (NSHL). Over the past decade, researches have mapped 92 loci and cloned 41 genes associated with non-syndromic hearing loss inherited in a Mendelian dominant, recessive, or X linked fashion (hereditary hearing loss homepage, http://www.uia.ac.be/dnalab/hhh/).

As the main components of bone and cartilage, collagens are related intimately to auditory function and, not surprisingly, mutations in collagens are found in many syndromic types of hearing loss. Included in this list are osteogenesis imperfecta (MIM 166200, 166220, 259420, *COL1A1*, and *COL1A2*), Alport syndrome (MIM 301050, 203780, 104200, *COL4A3*, *COL4A4*, and *COL4A5*), otospondylomegaepiphyseal dysplasia (OSMED syndrome)(*COL11A2*), and Stickler syndrome in both its classic (STL1, MIM 108300, *COL2A1*; STL2, MIM 604841, *COL11A1*) and non-ocular forms (STL3, MIM 184840, *COL11A2*), although the latter is more appropriately called heterozygous OSMED syndrome. Mutations in *COL11A2* also cause autosomal dominant non-syndromic hearing loss at the DFNA13 locus.[4]

The collagen family is comprised of 19 collagens encoded by at least 32 unique genes.[5] The common signature collagen motif is the sequential repetition of the amino acid triplet -Gly-X-Y-, where many of the X- and Y- positions are filled by the ring amino acids proline and hydroxyproline, facilitating the intertwining of three collagen polypeptide chains into a triple helix.[6] Type XI collagen is a minor collagen, accounting for less than 10% of total cartilage collagen. It functions as a spacer, maintaining the interfibrillar distance and fibril diameter of type II collagen.[7][8] The three α chain polypeptides of type XI triple helix are α1(XI), α2(XI), and α3(XI) and are encoded by *COL11A1* (1p21), *COL11A2* (6p21.3), and *COL2A1* (12q13.11–q13.2), respectively.[9]

All diseases associated with allele variants of *COL11A2* including non-ocular Stickler syndrome, OSMED syndrome and DFNA13, have a hearing impairment phenotype. The mutations of *COL11A2* associated with these diseases include missense mutations, nonsense mutations, small deletions, and insertions.[10–15] Phenotype–genotype comparisons suggest that the different phenotypes are mutation dependent. As collagen folding begins at the carboxy terminus where the nucleation domain is located, mutations close to the carboxy terminus generate a more severe phenotype.[16] This positional effect of missense mutations is common to other collagen associated diseases. For instance, a glycine to asparagine substitution in exon 22 of *COL2A1* causes lethal chondrodysplasia,[17] while the same amino acid substitution in exon 10 causes only isolated ocular abnormalities.[18] A nonsense or frameshift/stop mutation, in comparison to a missense mutation, is frequently silent in the heterozygote state.

Here we describe the identification of a missense mutation in *COL11A2* in a consanguineous Iranian family segregating autosomal dominant non-syndromic hearing loss (ARNSHL). The mutation does not affect a canonical glycine of the -Gly-X-Y- triplet repeat motif and neither is it close to the carboxy terminus. Our finding supports the hypothesis that the clinical phenotypes of *COL11A2* associated diseases are a reflection of mutation type and location.

METHODS
Patients and family
L-622 family members were ascertained in a combined otolaryngology/genetics clinic in Iran. A detailed history was taken to exclude conditions such as rubella, prematurity, drug use during pregnancy, perinatal trauma, sudden infant death syndrome, and meningitis. Persons with hearing loss underwent a complete physical examination, with ancillary tests as required. Otoscopy was done to exclude middle ear disease, followed by audiometry to quantitate the degree of hearing loss. On consenting persons, venous blood samples were obtained for DNA extraction. All procedures were approved by the institutional review boards of the University of Social Welfare and Rehabilitation Sciences, Tehran, Iran and the University of Iowa, Iowa City, Iowa USA.

Microsatellite genotyping in the L-622 family
A genome-wide scan was done using 400 fluorescent dye labelled microsatellite markers with an average spacing of 10

Abbreviations: ARNSHL, autosomal recessive non-syndromic hearing loss; OSMED, otospondylomegaepiphyseal dysplasia; PCR–RFLP, polymerase chain reaction–restriction fragment length polymorphism
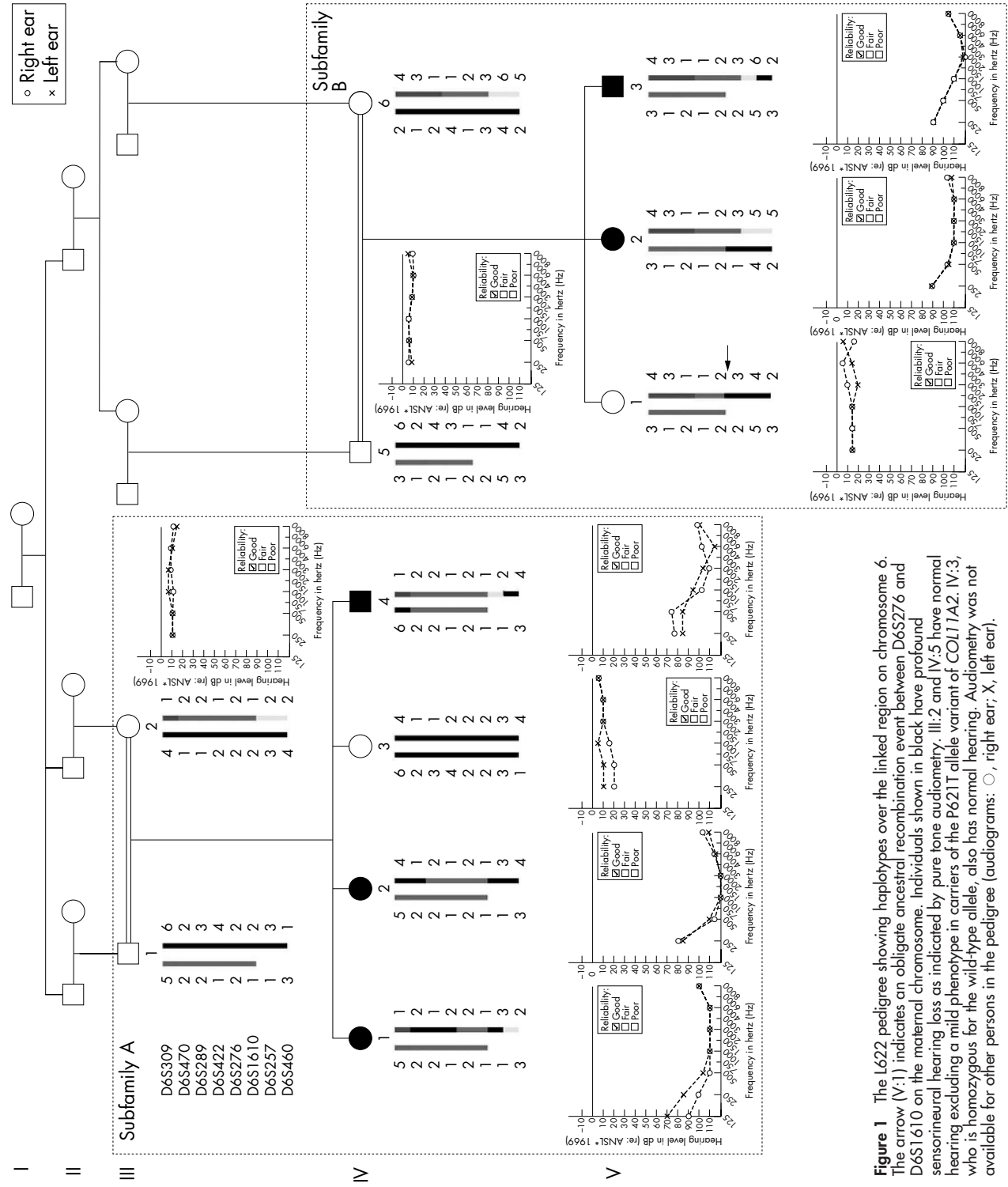
Figure 1 The L622 pedigree showing haplotypes over the linked region on chromosome 6. The arrow (V:1) indicates an obligate ancestral recombination event between D6S276 and D6S1610 on the maternal chromosome. Individuals shown in black have profound sensorineural hearing loss as indicated by pure tone audiometry. III:2 and IV:5 have normal hearing excluding a mild phenotype in carriers of the P621T allele variant of *COL11A2*. IV:3, who is homozygous for the wild-type allele, also has normal hearing. Audiometry was not available for other persons in the pedigree (audiograms: ○, right ear; X, left ear).

cM across the 22 autosomes and chromosome X (Prism Linkage Mapping Set, version 2.5; Applied Biosystems, Foster City, California, USA). Microsatellite markers were amplified by polymerase chain reaction (PCR) and analysed on an ABI Prism 3100 genetic analyser. Alleles were assigned using GeneMapper 3.0 software (Applied Biosystems). Haplotype reconstruction was determined with custom-made GeneScan software.

Mutation analysis by sequencing genomic DNA

All exons of *COL11A2* and their flanking sequences were amplified by PCR and sequenced using an ABI model 3700 automated sequencer. Sequence data were compared with published sequence of *COL11A2* using the Sequencher 4.1.4 software program package (Gene Codes Inc). Mutation position was assigned according to mRNA sequence from GenBank NM_080680 with nt 1 as first nucleotide of start codon.

PCR–RFLP genotyping of the missense mutation

Primers flanking exon 21 were used to PCR amplify genomic DNA of all participating L-622 family members and 269 Iranian controls. PCR products were digested with BsrI to yield either a 218 base pair (bp) fragment (non-mutant allele) or 116+102 bp fragments (mutant allele), which were resolved by 2% agarose gel electrophoresis.

GenBank accession numbers

Accession numbers included *COL11A2* DNA, U32169; mRNA, NM_080680; collagen α1(XI), NP_542196 (*Homo sapiens*), NP_031755 (*Mus musculus*); collagen α2(XI), NP_542411 (*Homo sapiens*), NP_034056 (*Mus musculus*), NP_997693 (*Rattus norvegicus*), and AAH43799 (*Xenopus laevis*).

RESULTS
Clinical phenotype

Clinical findings in family L622, a consanguineous Iranian family, were consistent with the diagnosis of ARNSHL (fig 1). All affected individuals had a history of prelingual profound hearing loss, with normal vestibular function as evidenced by age appropriate developmental motor milestones and physical tests of balance (Romberg test and heel–toe walking). Pure tone audiometry confirmed the presence of non-progressive profound sensorineural hearing loss (fig 1). Ophthalmological examination done by an ophthalmologist on all affected family members ruled out evidence of myopia, vitreo-retinal detachment, cataract, glaucoma, or other eye symptoms. An otolaryngological examination carried out by an otolaryngologist excluded midface hypoplasia and obvious palatal clefting, while palpation of the secondary palate excluded the presence of a submucous cleft palate. Stature was normal. In two of the five affected persons (V:2 and V:3) a general bone survey including the thoracolumbar spine, wrists, and knees showed no evidence of joint degeneration, abnormal epiphyseal development, or bone dysplasia. The profile of IV:2 showed mild mandibular retrognathia, consistent with a lateral skull radiograph, which additionally showed normal maxillary development and a class II dentoskeletal relationship. The father of IV:2 also had mild mandibular retrognathia (fig 2). Non-affected individuals had normal hearing as documented by pure-tone audiometry (fig 1).
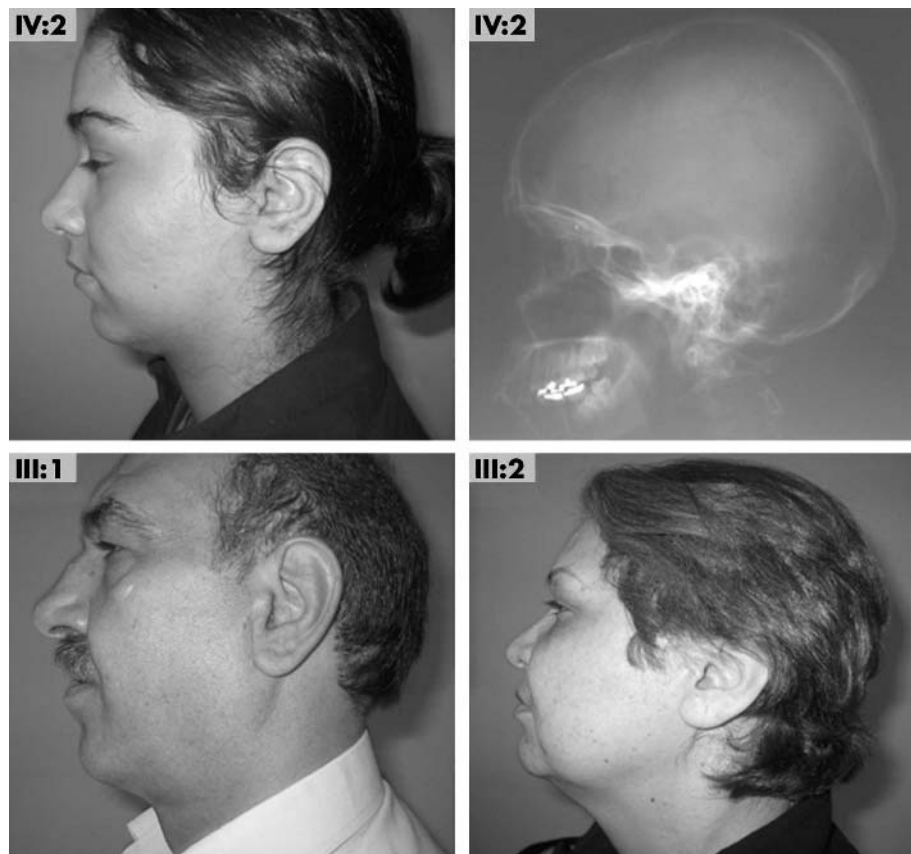

Figure 2 Lateral skull radiograph on IV:2 shows normal maxillary development, a class II dentoskeletal relationship, and mild mandibular retrognathia. Mandibular retrognathia is seen on a profile view and is present in III:1 but not in III:2 (parents of IV:2), both of whom are carriers of the P621T allele variant of *COL11A2*. (Photographs reproduced with patients' consent.)

Figure 3 Mutation analysis in the DFNB53 family. (A) Sequence data from IV:2 (congenitally deaf), V:1 (carrier of the P621T allele) and a control person. (B) Polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) data following BsrI digestion of amplified DNA. All affected individuals (−/−) have two digestion products of 116 bp and 102 bp; IV:3, not carrying the mutation, has the undigested product of 218bp; five carriers (+/−) have one undigested and two digested products. (C) Alignment of a portion of collagen type XI proteins from various species shows that P621 (arrow) in the collagen triple helical region is conserved across species (conserved amino acids, dark blue background; similar amino acids, light blue background; non-conserved amino acids, white background; boxed sequence, proline-rich region flanking the mutation site).

Localisation of DFNB53 to 6p21.3

We completed a genome-wide linkage analysis using 400 polymorphic microsatellite markers (Applied Biosystems) on 11 members of family L622 (fig 1). Genotyping data from subfamily A identified a novel locus for ARNSHL on chromosome 6p21.3 flanked by D6S422 (telomeric) and D6S257 (centromeric), which was designated DFNB53. Individual V:1 further narrowed the DFNB53 interval to a 9.4 cM region flanked by D6S276 (telomeric) to D6S1610 (centromeric).

Mutation analysis in family L622

The 9.4 cM DFNB53 interval includes 246 known genes, including *COL11A2*. Because mutations in *COL11A2* cause

deafness at the DFNA13 locus, we considered it an excellent functional candidate and completed mutation screening of its 66 exons. We identified a cytosine to adenine transition in exon 21 (nt 1861) in the heterozygous state in individual V:1 and in the homozygous state in individual IV:2 (fig 3A). Further analysis by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis showed all five affected persons were homozygous for this variation, while the four parents and V:1 were mutation carriers (fig 3B). Genotyping data from 269 Iranian controls (538 chromosomes) did not show the same variation, suggesting that it is rare in the general population. The resulting change at the amino acid level—P621T based on UniProt P13942—affects the Y position of the -Gly-X-Y- repeat near

Table 1 Reported allele variants of *COL11A2*

Type of mutation	Mutation — Homozygous	Mutation — Heterozygous	Predicted consequence	Phenotype	Inheritance	Proposed mechanism of action	Reference
Nonsense mutation or frameshift stop	732delC		Frameshift/stop in exon 6	OSMED	Recessive	Functionally null allele secondary to protein truncation or non-sense mRNA decay	12
	2492C→A		S831X(345)	OSMED	Recessive		12
	2406_2409del, 2405_2410ins9bp		Frameshift/stop in exon 32	OSMED	Recessive		12
	3991C→T		R1331X (845)	OSMED	Recessive		10
	4821_4843del		Frameshift/stop in exon 64	OSMED	Recessive		12
	1636C→T and IVS22-2 A→G*		R546X (60) and in-frame deletion of exon 23	OSMED	Recessive		12
	3032_3033insC and 4750G→T*		Frameshift/stop in exon 43 and G1584X (1098)	OSMED	Recessive		12
	3991C→T and IVS53+5G→A*		R1331X (845) and frameshift/stop in exon 56	OSMED	Recessive		12
		529C→T‡	R177X	Non-syndromic cleft palate	‡		19
In-frame deletion		2775_2801del	In-frame deletion of 27 bp in exon 39	Non-ocular Stickler	Dominant	Functional allele with dominant-negative effect on collagen folding	13
		4135C→T	In-frame deletion of exon 57	Non-ocular Stickler	Dominant		11
		IVS60-1G→A	In-frame deletion of exon 60	Non-ocular Stickler	Dominant		15
Missense mutation	1861C→A†		P621T	Non-syndromic hearing loss	Recessive	Functional allele with recessive-negative effect on collagen folding	
	1982G→A		G661R (175)	OSMED	Recessive		15
		2423G→A	G808E (323)	Non-syndromic hearing loss	Dominant	Functional allele with dominant-negative effect on collagen folding	4
		3100C→T	R1034C (549)	Non-syndromic hearing loss	Dominant		4
		4322G→A	G1441E (955)	WZS/non-ocular Stickler	Dominant		14

Mutation positions are assigned according to collagen α2(XI) chain precursor (UniProt P13942); numbers in parenthesis are used in previous studies.
*Compound heterozygotes.
†Mutation identified in this study.
‡Reported in the heterozygous state in a person with non-syndromic cleft palate deformity; the father of the proband also carries R177X but has no cleft palate deformity, suggesting either variable penetrance or another aetiology for the isolated non-syndromic cleft palate deformity in this family.
OSMED, otospondylomegaepiphyseal dysplasia; WZS, Weissenbacher–Zweymuller syndrome (similar to non-ocular Stickler syndrome).
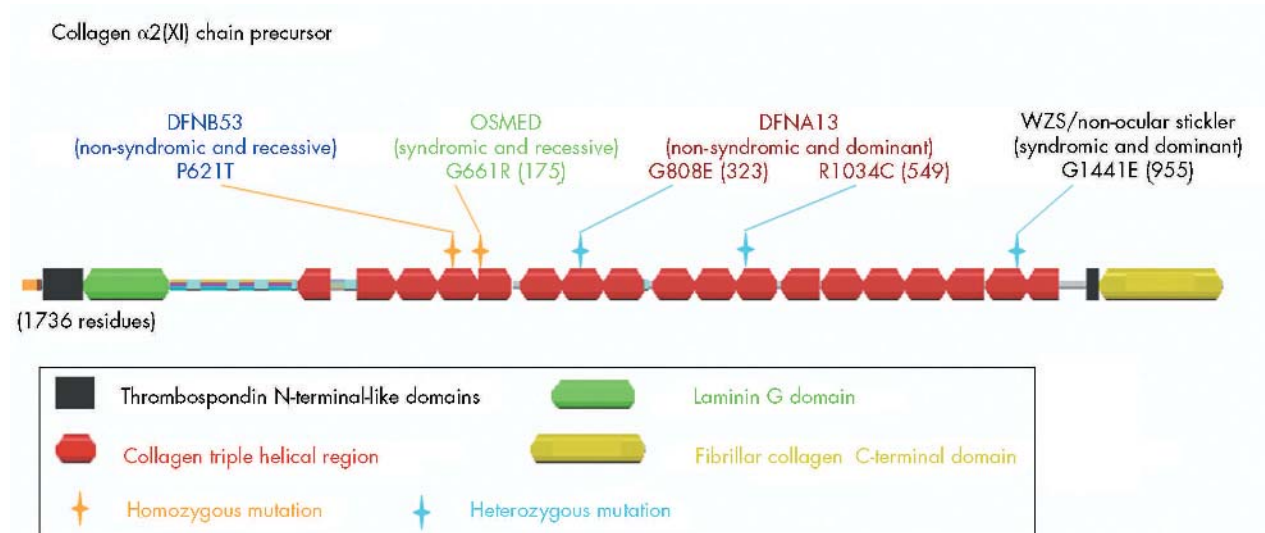
Figure 4 The location of reported missense mutations in collagen α2(XI) chain precursor. Graphic view of the domain structure is from the Protein Families Database (UniProt P13942). Note the relation between mutation position/type and phenotype. Carriers of the DFNB53 and OSMED causing mutations have a normal phenotype. Numbers in parentheses indicate amino acid positions in mature collagen α2(XI) protein and were used in previous publications.

the amino-terminus of triple helical region. Sequence comparison of collagen type XI α1 and α2 peptides across species identifies the replaced proline as an evolutionarily conserved amino acid (fig 3C).

Analysis of reported *COL11A2* mutations

Several mutations in *COL11A2* have been reported, including 10 nonsense or frameshift/stop mutations in patients with recessive OSMED syndrome,[10 12] three in-frame deletions in dominant non-ocular Stickler syndrome, and four missense mutations that lead to either dominant or recessive disease[4 14 15] depending on the type and location of the mutation (fig 4; table 1).

DISCUSSION

Type and location of *COL11A2* mutations determine disease phenotype

Mutations of *COL11A2* cause syndromic and non-syndromic autosomal dominant and recessive hearing loss.[10 11 13–15] This phenotypic spectrum is the consequence of both mutation type and location. Syndromic disease is associated with either a dosage or a dominant negative effect. The former is seen with nonsense or frameshift/stop mutations that are associated with nonsense mediated mRNA decay or generate short lived but rapidly degraded truncated proteins.[20] In the heterozygous state, these mutations cause haploinsufficiency but no disease phenotype, while in the homozygous state, they result in absence of α2(XI) and autosomal recessive OSMED syndrome. In-frame deletions in *COL11A2*, in contrast, are translated into shortened mutant proteins that are incorporated, albeit abnormally, into the collagen triple helix. The phenotypic consequence is dominantly inherited non-ocular Stickler syndrome and reflects a dominant negative mechanism of action.

Missense mutations of *COL11A2* cause both syndromic and non-syndromic hearing loss. Of the five reported missense mutations, three change the core amino acid glycine of the -Gly-X-Y- consensus repeat (fig 4; table 1). Because G1441E is closest to the carboxy terminus, which is also the site of trimerisation initiation of the collagen helix, this glycine mutation has the most severe phenotypic consequence of the three—it causes autosomal dominant Weissenbacher–Zweymuller syndrome (MIM 277610, non-ocular Stickler syndrome). G808E causes only autosomal dominant hearing loss, and G661R causes autosomal recessive OSMED syndrome.

Hypothesised effect of the P621T mutation

The homozygous mutation we identified, P621T, differs from reported *COL11A2* mutations in two respects: it does not involve glycine and it is not close to the carboxy terminus. We hypothesize that this variant is pathological because it either destabilises the triple helix or affects collagen metabolism.

Proline always occupies the X or Y position in collagen and often undergoes post-translational hydroxylation, a modification that stabilises the triple helix. P621T could destabilise the triple helix in two ways. First, isoleucine-620 could repress glycosylation of threonine-621, and when non-glycosylated, threonine cannot stabilise the triple helix.[21] In addition, because the region is proline-rich and therefore able to form only weak hydrogen bonds, a proline substitution may destabilise adjacent amino acid interactions[22] (fig 3C).

The P621T substitution also could affect collagen α2(XI) metabolism. Enzymatic degradation of type XI collagen may be important in disease states.[23] Matrix metalloproteinase 2 (MMP2) degrades type XI collagen by recognising Gly–Iso or Gly–Leu bonds[24]; however, Gly–Iso followed by Pro is resistant to collagenase.[25] Increased activity of MMP2 destroys collagen architecture.[26] Thus it is possible that threonine-621 in mutant collagen type XI α2 permits abnormal degradation of collagen type XI by MMP2 induced cleavage.

Conclusions

We report the first mutation in *COL11A2* associated with ARNSHL (homozygosity for P621T in a consanguineous Iranian family). This missense mutation is close to the amino-terminus. Based on comparative genotype–phenotype studies of all *COL11A2* mutations, which show that mutation type and location are important predictors of phenotype, and we hypothesise that P621T alters collagen folding or degradation.

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