Novel Variants in COL4A3 and COL4A4 are Causes of Alport Syndrome in Rio Grande do Norte, Brazil

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Abstract

Background. Alport syndrome is a progressive and hereditary nephropathy, characterized by hematuria and proteinuria, and extrarenal manifestations as hearing loss and eye abnormalities. The disease can be expressed as autosomal recessive or dominant, caused by variants in COL4A3 and COL4A4 *loci*, respectively, or X-linked caused by variants in COL4A5 *locus*.

Methods. Two unrelated families with Alport Syndrome from northeast of Brazil were studied and whole exome sequencing were performed. DNA sequences were mapped against the human genome (GRCh38/hg38 build) to identify associated mutations.

Results. Variant analysis showed deleterious variants in COL4A3 and COL4A4 *loci* in chromosome 2. Two variants were detected with alternative alleles in a homozygous state in the probands. One novel premature stop codon at position 481 of COL4A3 *protein* is present in one family and one frameshift mutation leading to a premature stop codon at position 786 of COL4A4 protein in the other family. Both Alport cases presented their variants surrounded by a broad runs of homozygosity (ROH).

Conclusions. The autosome recessive inheritance coupled with the runs of homozygosity in both families suggest inbreeding.

Keywords: Alport Syndrome, whole exome sequencing, runs of homozygosity, kidney diseases, collagen type IV

Introduction

Alport syndrome is a hereditary Mendelian disease in which individuals progress with hematuric nephritis and sensorineural deafness. These symptoms are the results of defect in the glomerular basement membranes biogenesis [1]. The pathophysiology of Alport Syndrome consists of structural defects in type IV collagen alpha chains that constitutes the glomerular basement membrane [2-3]. Collagen type IV are encoded by six distinct genes (COL4A1 to COL4A6) consisting of six alpha chains ($\alpha 1$ - $\alpha 6$) which assemble in heterotrimers (three α chains coiled together) forming a protomer [4-5]. There are three major mutations in $\alpha 3$, $\alpha 4$ or $\alpha 5$ generating defective type IV collagen networks and preventing proper structure of glomerular basement membrane [6-7].

Although there are six alpha chains, only three different protomers are known, $\alpha 1\alpha 1\alpha 2$ (IV), $\alpha 5\alpha 5\alpha 6$ (IV) and $\alpha 3\alpha 4\alpha 5$ (IV) [8-10]. Those protomers form the type IV collagen networks which are an essential part of the extracellular matrix and the structure of all basement membrane. Type IV protomers consists in a triple collagenous helix domain of about 400nm, characterized by a triplet amino acid repeat sequence of Gly-X-Y. Furthermore, a globular non-collagenous domain is found in the 3' as well as a short domain, called 7S in the 5' end. These domains form a dense collagens IV network, once secreted in the extracellular matrix [11-12].

The $\alpha 1\alpha 1\alpha 2$ (IV) protomer is ubiquitously distributed in all basement membranes during embryogenesis, later are replacing by the protomer $\alpha 3\alpha 4\alpha 5$ (IV) in glomerular basement membrane and protomer $\alpha 5\alpha 5\alpha 6$ in other tissues [13]. The $\alpha 3\alpha 4\alpha 5$ (IV) is known to be more cross-linked protomer [14-15], resulting in a stronger and more resistant heterotrimer [16] Collagen IV network has a crucial role for development of glomerular basement membrane during ontogenesis, which helps to support tissue

integrity besides cell signaling, morphogenesis and tissue regeneration [4]. There is an interaction in the biogenesis of collagen produced by podocytes and the glomerular basement membrane. Moreover, $\alpha3\alpha4\alpha5$ (IV) protomer is also produced in other tissues, such as the cochlea and eyes [17-18].

There are several variants described for the COL4A3 (I120070, OMIM) loci and COL4A4 (120131, OMIM) *loci*, localized in 2q35-37, and COL4A5 (303630, OMIM) *locus*. Autosomal recessive Alport Syndrome is related to modifications in the $\alpha3$ (IV) and $\alpha4$ (IV) chains, whereas X-linked Alport Syndrome is related to modifications in the $\alpha5$ (IV). In the case of $\alpha6$ chain, although the $\alpha5\alpha5\alpha6$ (IV) protomers can be formed in autosomal recessive Alport Syndrome. Experimental study demonstrated that this gene is not associated with Alport Syndrome [19]. Both modifications affect the biogenesis of type IV collagen and lead to a reduction or absence of these molecules in glomerular basement membrane progressing to a renal failure [20].

Inbreeding can increase the risk of Alport syndrome [21]. Recent studies have shown an increase of genomic homozygosity with increasing consanguinity [22-26]. In this work, we report the results of Alport syndrome cases from two families from the Northeast region of Brazil. We identify two novel variants (stop codon gained and frameshift) and the mutations result in a truncated $\alpha3$ and $\alpha4$ chains. In addition, there is a broad region of runs of homozygosity (ROH).

Material and Methods

Biological material from patients (Families Sampling)

Two unrelated families, Family 1 (F1) and Family 2 (F2), of the Northeast region in Brazil (Rio Grande do Norte state) were evaluated. Nuclear Family 1 had 4 cases of Alport, but within the extended family there were 5 other cases, all had been transplanted and Family 2 apparently had a sporadic case of Alport.

Ethical Considerations

The study protocol was reviewed and approved by the Federal University Ethical Committee (CEP-UFRN 50-01) and by the Brazilian Ethical Committee (CONEP-4569). All participants and-or legal guardians read, approved and signed the informed consent. Subjects with medical conditions discovered during the study were treated by the study team or referred or taken to the appropriate medical resource in Natal. Subjects with Alport syndrome had already been transplanted at the time of recruitment and they were been followed at the University Hospital for their medical care.

DNA extraction

DNA was extracted from 10 ml of anticoagulated whole blood (EDTA) by erythrocyte lysis in 70 µg of $\text{NH}_4\text{H}_2\text{CO}_3/\text{ml}$ and 7.0 mg of $\text{NH}_4\text{Cl}/\text{ml}$ followed by lysis of leukocytes in 1% sodium dodecyl sulfate, 100 mM EDTA, and 200 mM Tris (pH 8.5) and precipitation in isopropanol. DNA sample underwent quality control which included gel electrophoresis and quantification and determination of integrity by fluorometer (qubit, Thermo Fisher, USA).

Whole Exome Sequencing

In each family the father, mother, unaffected sibling and proband (n= 4) had their exomes sequenced. Whole exome sequencing (WES) was performed by the University of Iowa Genomics Division using manufacturer recommended protocols. Briefly, 3µg of genomic DNA were sheared using the Covaris E220 sonicator. The sheared DNA was used to prepare indexed whole exome sequencing libraries using the Agilent SureSelect XT Human all exon v6 + UTR kit (Agilent Technologies, Santa Clara, CA, Cat. No. 5190-8883). The molar concentrations of the indexed libraries were measured using the Fragment Analyzer (Agilent Technologies) and combined equally into pools for sequencing. The pooled libraries were loaded on an Illumina HiSeq 4000 genome sequencer using the 2 x 150bp paired-end sequencing by synthesis (SBS) chemistry.

Raw Reads Preprocessing and Reference Genome Mapping

FastQC (v0.11.4) [27] software was used to quantify the sequences and to analyze the sequencing quality. Adapters was removed by Trim Galore (v0.4.1) [28] using the option to execute Cutadapt (v1.8.3) [29], with default values. Reads were mapped using BWA (v0.7.12-r1039) [30] software, with mem mode against to GRCh38/hg38 build [31]. Functions stats and depth from SAMtools (v1.7) [32] were used to calculate total reads mapped, total exome mean coverage and mean coverage in ROH and remaining regions. Normalized mean coverage was calculated dividing the average coverage per base by the individual number of reads sequenced then multiplying by the average of reads sequenced of all individuals. Also, mpileup function from SAMtools was used to generate input to VarScan2 (v2.3.9) [33] software.

Runs of Homozygosity (ROH) ratio

To obtain total chromosomal ROH lengths the algorithm H3M2 [34] was used followed by a filter of long ROH fragments lengths (1.5Mb). The software was applied in

the mapping file for each individual sample of this study. A reference length of ROH was determined by applying H3M2 with the same filter in 20 random publicly available CEU samples sequenced by the 1000 Genomes Project [35]. Thus, as a measure of the consanguinity/endogamy a homozygous ratio was calculated dividing the total ROH length of each family member by the average of ROH length from CEU samples.

Variant Calling and Data Refinement

Best practices from Genome Analysis ToolKit (v3.4-46) HaplotypeCaller [36] (GATK-HC) were performed with *Base Quality Score Recalibration* (BQSR) mode using the following known sites: The Single Nucleotide Polymorphism Database (dbSNP 146); Mills & 1000G gold standard indels for hg38 and 1000G phase1 SNPS high confidence sites for hg38. Vcftools (v0.1.13) [37] was used to filter low coverage variants and to maintain records with at least one sample with variant call data. Variants with minDP < 20 and max-missing > 0.125 were eliminated from the analysis. Vcfstats [38] from vcflib package was used to summarize basic variant calling statistic.

Homozygosity Mapping, Variant Annotation and Haplotype Phasing

To detect differences between the homozygous and heterozygous signals Homozygous Stretch Identifier - HomSI (v2.1) [39] - software was used with default parameters values. Databases such as Exome Aggregation Consortium [40], 1000 Genomes Project and dbSNP 146 were integrated and variants were annotated by ANNOVAR (v2018Apr16) [41] and SnpEff (v 4.1) [42] software, respectively. Missing data inference and haplotype phasing were performed using Beagle (v5.1) [43] software.

Identification of deleterious variants

The analysis focused on identifying homozygous pathogenic variants. We restricted the exome sequence data to variants with 1) allelic frequencies < 1% in Exome Aggregation Consortium database; 2) gene and protein expression (> 1 FPKM) in kidney, cochlea and eye from Illumina Body Map, The Human Protein Atlas (HPA) and Genotype-Tissue Expression Project database; 3) clinical significance from ClinVar database. Variants with a structural effect (frameshift and stop codon gained) were considered deleterious.

Results

Genotyping and exome Screening

On average 98.6% of the exome-wide was covered with at least 20-fold coverage at ~190x of coverage (Supplementary Table S1). A total of 106,726 (97,327 SNVs, 9 MNPs and 9,390 INDELS) variants were reported and Ti/Tv ratio was equal to 2.4. From those, 16,830 variants were nonsynonymous, 124 stop codons gained and 262 were frameshifts.

A total of 12 deleterious variants were identified (1 stop codon gained and 11 frameshift) with alternative allele homozygous state exclusively in probands (Supplementary Table S2). From these 12 variants, 8 variants had an allele frequency greater than 1% in the Exome Aggregation Consortium database including one variant classified as benign clinical significance in the clinvar database; 2 variants were not expressed in any of the organs involved in Alport disease. Two of the 12 variants were classified as possibly pathogenic and present in genes expressed in the kidney, eye and cochlea. The two later variants are directly linked to collagen type IV production and present in COL4A3 (p.Try481*) and COL4A4 (p.Val741fs) *loci*. No deleterious variants were found in COL4A5.

Variants along COL4A genes

Twenty-three variants (Supplementary Table S3) were present in COL4A genes. Twelve variants were presents in COL4A4 (6 synonymous, 5 non-synonymous and 1 frameshift) and eleven in COL4A3 (3 synonymous, 7 non-synonymous and 1 stop gain). Figure 1 shows the distribution of all nonsynonymous and deleterious variants in this study in COL4A4, COL4A3 and COL4A5 loci. Only stop gained and frameshift variants

were not reported in the ClinVar database. All other variants were reported on Clinvar as benign for cases of Alport Syndrome.

Eleven variants in COL4A3 occurred at triple helix region domain of collagen type IV. We observed a premature stop codon gained (p.Try481*) in all members among Family 1. In this family, proband presented this variant with alternative allele in homozygous state. Other individuals of this family had the same variant at heterozygous state. All members of Family 1, except the proband, have the same genotype in this locus, whereas members of Family 2 do not have this variant.

Nine variants in COL4A4 occurred at triple helix region domain of collagen type IV. We observed a frameshift (p.Val741fs) in all members among Family 2. In this family, proband had a homozygous frameshift leading to a truncated protein with 786 amino acids. Other members of this family had the same frameshift at heterozygous state. Members of Family 1 do not have this variant.

Segregation analysis in COL4A genes

Haplotype phasing showed offspring's alleles were inherited together from copies of paternal and maternal chromosomes. Both probands presented an excess of nonsynonymous variants at homozygous state. For Family 1, proband had four variants in COL4A3 and all remain members had the same three variants in COL4A3. Conversely, Family 2 proband had four variants in COL4A3 and three in COL4A4 loci while all remain members had at most three variants distributed in both COL4A genes. All variants of the genes encoding $\alpha 4$ and $\alpha 3$ chains of collagen type IV perfectly co-segregated with the disease in both families (Figure 2). All individuals showed approximately 190x (Table 1) of coverage in homozygous regions and remaining regions.

Non-synonymous variant alleles found in COL4A genes, when analyzed individually, occurred at high allelic frequency rates in the 1KGP database. However, their co-occurrence with haplotype frequency was rare in all cases. Co-occurrence including deleterious variants presented in this study did not occur in any 1KGP individual.

Homozygous regions in COL4A genes

Chromosome 2 showed a common ROH locus in both probands (Figure 3). In Family 1 proband has a homozygous locus of 20Mb with 140 genes. In Family 2 proband has a homozygous locus of 75Mb with 364 genes. By analyzing this region, we observed that only the COL4A3 and COL4A4 genes had deleterious variants.

An average of 116,876,596 bp of ROH length for CEU population (Supplementary Table S4). The total of ROH fragments, ROH length for all autosomal chromosomes and homozygous ratio for each member of the family (Table 2 and 3) showed high level of consanguinity, with high homozygous ratio in the offspring. Family 1 had a homozygous ratio of 1.05, 2.15, 3.53 and 2.03 for the father, the mother, the unaffected son and the proband respectively. Family 2 had a homozygous ratio of 0.70, 0.56, 3.15 and 2.09 for father, mother, unaffected son and proband respectively. This result highlighted a relation with the number of long fragments of ROH and homozygous ratio: the higher values of fragments, the higher values of homozygous ratio.

Discussion

In the present study, we investigated two families from northeast of Brazil with Alport Syndrome and we report two novel variants responsible for the disease. The first one was a novel nonsense mutation in COL4A3 gene in proband from Family 1. The second one was a frameshift in COL4A4 locus in proband from Family 2. Both findings occurred in triple helix domain and had a broad region of ROH covering both genes in probands. Cosgrove and Liu [8] states that stop codon gained, frameshifts and other mutations that result in chain termination are associated with a more severe phenotype.

Szpiech et al [44] using data obtained by whole exome sequencing demonstrated that a significantly larger fraction of all predicted harmful homozygous variants are surrounded by ROH stretch when compared to those corresponding fractions of non-damaging homozygous variants. Long ROH (> 1.5 Mb) are known to be favored by inbreeding and IBD inheritance processes and characterized by an enrichment of deleterious recessive mutations [45, 46]. Most recessive disease variants are identified via homozygosity mapping. Using high-density genome-scan data in European populations McQuillan et al [24] indicate that the numbers and lengths for ROHs above 1.5Mb are clearly relation to endogamy and isolation behind the effects on ROHs, as occurred in our Alport syndrome.

A homozygous region was present in both probands in chromosome two but being broader in Family 2 when compared to Family 1 (Figure 2). Although unaffected offspring had the highest value for homozygous ratio (Table 2 and Table 3), in both families, type IV collagen is not affected. A possible explanation for this excessive homozygosity in the loci region of COL4A3 and COL4A4, would be a large deletion occurring in the probands leading to uniparental inheritance. However, the phasing of chromosomes as well as the

average of coverage in ROH and remaining regions (Figure 2 and Table 1) pointed out to an allele inheritance and not to a deletion inheritance rejecting a hypothesis of deletion event. Recent studies [47, 48] reported that mutations in COL4A3 and COL4A4 *loci*, when in *cis*, can lead to a form of benign familial hematuria, in a similar genetic way as Alport syndrome. Benign familial hematuria is considered as a phenotypic expression and represents a carrier state for autosomal recessive Alport syndrome.

All family 1 members have a nonsense variant (p.Tyr481*) in COL4A3 gene, although only the proband has it with alternative allele at homozygous state. This variant is novel. In addition, it is known that COL4A3 nonsense phenotype is severe and associated with end-stage renal disease, deafness and ocular lesions [11]. As reported in this study, the observed stop codon gained falls in collagenous domain of type IV collagen leading to a truncated protein, and consequently destabilizing collagen IV network formation. Other mutations may increase the risk of AS because they had perfectly co-segregated with disease. In this family we observed all members having high homozygosity rate.

Members of family 2 had a frameshift variant (p.Val741fs) in COL4A4 gene, but only the proband has with alternative allele at homozygous recessive state (see Table 1). Frameshift is the most important evidence for Alport Syndrome heritage in this family due to the insertion of one base in the reading frame and led to a truncated protein with 786 residues. Thus, herein we propose Alport Syndrome resulting in phenotype of proband in this family. Zhu *et al* [49] obtained a similar result using next generation sequencing and identified a frameshift leading to truncated COL4A4 protein with 1634 amino acids and considering as a loss of function variant. In this family we noticed only the offspring had

high homozygosity rate. This reflects origin from a small isolated population and presents features such as population genetic historical and geographical factors.

Conclusions

This work describes two novel variants responsible for Alport cases in two families from northeast of Brazil. Those variants were searched in the databases of 1000 genomes and Clinvar and they are rare alleles, not yet identified in the world population. The two pathogenic variants identified are located on an ROH locus. Interestingly both Alport probands have ROH in the COL4A3 and COL4A4 loci, and all variants are homozygous. There is a need to screen people who have lost kidney function for genetic mutations, allowing a better understanding of Alport Syndrome and their respective penetrance. In addition, it is important to identify people heterozygous for variants associated with Alport Syndrome in areas with high level of inbreeding.

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Figure 1. Distribution of deleterious and homozygous non-synonymous variants in COL4A4, COL4A3 and COL4A5 genes.

Supplementary Table S1: Exome-wide base coverage summary

Sample	Total Mapped reads	Total exome mean coverage (normalized)	Total Covered Bases (20x fold)	% 20x fold bases covered
Father (F1)	157,896,820	194.15	89,152,408	98.54
Mother (F1)	180,487,311	189.62	89,312,826	98.71
Son (F1)	204,975,749	184.55	89,624,633	99.06
Proband (F1)	147,647,415	191.20	88,816,063	98.16
Father (F2)	212,010,435	191.23	89,699,378	99.14
Mother (F2)	187,140,367	193.47	89,334,742	98.74
Son (F2)	185,054,786	194.51	89,003,689	98.37
Proband (F2)	153,688,254	195.54	88,989,473	98.36

Supplementary Table S2: Possibly deleterious variants with alternative allele at homozygous state in probands and at heterozygous state in remains members of the family															
Proband's Fam	Position	dbSNP_ID	Gene	Variant Effect	Clin Var	ExAC allele frequency	RNASeq Kidney expression*	HPA Kidney Expression*	GTEx Kidney Expression*	RNASeq Retina Expression*	HPA Retina Expression*	GTEx Retina Expression*	RNASeq Ear Expression*	HPA Ear Expression*	GTEx Ear Expression*
1	Chr2:174427852	rs145699077	SCRN3	FRAME SHIFT	NA	0.2799	9	19.2	16	0	0	0	0	0	0
1	Chr2:227267027	NA	COL4A3	STOP GAINED	NA	0	14.44	22.9	47	0	0	0	0	0	0
1	Chr15:98968575	rs5814919	PGPEP1L	FRAME SHIFT	NA	0.5109	0.09	0	6	0	0	0	0	0	0
2	Chr2:227059568	rs141127013	COL4A4	FRAME SHIFT	NA	0	9	10.7	35	0	0	0	0	0	0
2	Chr7:100773854	NA	ZAN	FRAME SHIFT	NA	0	0.005625	0	0.24	0	0	0	0	0	0
2	Chr7:100787939	rs72364644	ZAN	FRAME SHIFT	NA	0.3732	0.005625	0	0.24	0	0	0	0	0	0
2	Chr11:4769643	rs34672924	OR51F1	FRAME SHIFT	Benign	0.2129	0	0	0.74	0	0	0	0	0	0
2	Chr11:60397879	rs3217518	MS4A14	FRAME SHIFT	NA	0.6394	0.25	0	10	0	0	0	0	0	0
2	Chr17:40701882	rs11309872	KRT24	FRAME SHIFT	NA	0.4839	0.16	0	2	0	0	0	0	0	0
2	Chr17:81647906	NA	TSPAN10	FRAME SHIFT +	NA	0	0.04	0	6	0	0	0	0	0	0

STOP GAINED															
2	Chr19:1004897	rs10666583	GRIN3B	FRAME SHIFT	NA	0.2448	0.09	0	10	0	0	0	0	0	0
2	Chr19:52383892	rs34470614	ZNF880	FRAME SHIFT	NA	0.4718	4.84	6.2	13	0	0	0	0	0	0

*expressed values are in Fragments per kilo base per million mapped reads (FPKM).

Supplementary Table S3: Coding variants found by NGS in COL4A4 and COL4A3 genes in chromosome 2.

Chr2:Position	SNP_ID	Mutation Type	Proband	Son	Mother	Father	Proband	Son	Mother	Father	Gene	Amino acid change	ExAC	ClinVar	ClinVar Reported as Alport Condition?
			(F1)	(F1)	(F1)	(F1)	(F2)	(F2)	(F2)	(F2)					
227007466	rs2228557	SYNONYMOUS	G G	G G	G A	G G	G G	G A	G A	G A	COL4A4	p.Phe1644Phe	0.4657	Benign	Yes
227008279	rs2228555	SYNONYMOUS	T T	T T	T C	T T	T T	T C	T C	T C	COL4A4	p.Val1516Val	0.5602	Benign	Yes
227022057	rs3752895	NON SYNONYMOUS	A A	A A	A G	A A	A A	A G	A G	A G	COL4A4	p.Ser1403Pro	0.4763	Benign	Yes
227027903	rs2228556	SYNONYMOUS	C C	C C	C T	C C	C C	C T	C T	C T	COL4A4	p.Pro1360Pro	0.4700	Benign	Yes
227027942	rs16823077	SYNONYMOUS	T T	T T	T T	T T	T T	T C	T T	T C	COL4A4	p.Leu1347Leu	0.0070	Benign	Yes
227032170	rs2229812	SYNONYMOUS	C C	C C	C T	C C	C C	C T	C T	C T	COL4A4	p.Lys1228Lys	0.4716	Benign	Yes
227032260	rs10203363	SYNONYMOUS	C C	C C	C T	C C	C C	C T	C T	C T	COL4A4	p.Gly1198Gly	0.4746	Benign	Yes

227051116	rs1800517	NON_SYNONYMOUS	G G	G G	G A	G G	G G	G A	G A	G A	COL4A4	p.Pro1004Leu	0.5205	Benign	Yes
227052374	rs80243096	NON_SYNONYMOUS	T T	T T	T T	T T	C C	C T	C T	C T	COL4A4	p.Ile967Val	0.0073	Benign	Yes
227059568	rs14112701 3	FRAME_SHIFT	A A	A A	A A	A A	AG AG	AG A	AG A	AG A	COL4A4	p.Val741fs	.0.0000	Not Reported	No
227089883	rs2229814	NON SYNONYMOUS	G G	G G	G A	G G	A A	A A	A A	A A	COL4A4	p.Pro482Ser	0.5344	Benign	Yes
227147467	rs16823264	NON SYNONYMOUS	A A	A A	A A	A A	A A	A G	A G	A G	COL4A4	p.Ile6Thr	0.0285	Benign	Yes
227238007	rs13424243	NON SYNONYMOUS	G G	G C	G C	G C	C C	C C	C G	C C	COL4A3	p.Gly43Arg	0.2884	Benign	Yes
227246719	rs10178458	NON SYNONYMOUS	C C	C C	C C	C C	C C	C T	C C	C T	COL4A3	p.Leu141Pro	0.8302	Benign	Yes
227248459	rs6436669	NON SYNONYMOUS	G G	G G	G G	G G	G G	G A	G G	G A	COL4A3	p.Glu162Gly	0.8304	Benign	Yes
227263824	rs10205042	SYNONYMOUS	T T	T T	T T	T T	T T	T T	T C	T T	COL4A3	p.Leu399Leu	0.7667	Benign	Yes
227266453	rs11677877	NON SYNONYMOUS	A A	A A	A A	A A	A A	A G	A A	A G	COL4A3	p.His451Arg	0.0944	Benign	Yes
227267027	.NA	STOP_GAINED	A A	A T	A T	A T	T T	T T	T T	T T	COL4A3	p.Tyr481*	0.0000.	Not Reported	No
227267036	rs34019152	SYNONYMOUS	G G	G G	G G	G G	G G	G A	G G	G A	COL4A3	p.Gly484Gly	0.0923	Benign	Yes
227270915	rs28381984	NON SYNONYMOUS	T T	T T	T T	T T	T T	T C	T C	T C	COL4A3	p.Pro574Leu	0.4694	Benign	Yes
227283825	rs75519005	SYNONYMOUS	C C	C C	C C	C C	C C	C T	C C	C T	COL4A3	p.Pro905Pro	0.0051	Benign	Yes
227298737	rs57611801	NON SYNONYMOUS	C C	C C	C C	C C	C C	C C	C A	C C	COL4A3	p.Asp1269Glu	0.0441	Benign	Yes

227304032	rs73996414	NON SYNONYMOUS	C C	C C	C C	C C	C C	C C	C A	C C	COL4A3	p.Asp1347Glu	0.0078	Benign	Yes
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Figure 2. COL4A4 and COL4A3 alleles co-segregating. Figure indicates the variants of chromosome 2 for COL4A4 and COL4A3 of all individuals in families, in homozygosity (non-synonymous variant in red, homozygous non-synonymous variant in yellow). The probands are in black. Black * defines a stop codon gain in proband of Family 1 and a frame-shift in proband of Family 2.

Table 1: Normalized mean coverage by samples in ROH and remaining regions for complete chromosome 2 extension

Sample	Total Mapped reads to chr2	Normalized mean coverage in homozygous region	Normalized mean coverage in remaining regions
Father (F1)	11,361,323	191.45	195.62
Mother (F1)	12,778,526	186.90	190.10
Son (F1)	14,695,917	183.38	184.14
Proband (F1)	10,429,522	189.51	191.46
Father (F2)	15,160,228	192.15	191.13
Mother (F2)	13,382,947	198.62	192.09
Son (F2)	13,375,114	198.19	194.00
Proband (F2)	11,124,247	200.07	194.61

Figure 3. Differences between homozygous and heterozygous signals in complete chromosome 2 extension. A) Family 1, B) Family 2. Black arrow shows where homozygosity region begins in both probands (dark blue line). Red and green * define COL4A4 and COL4A3 genes respectively.

Table 2. Number and total length of ROHs of the Family 1.

Sample	Number of ROHs (cut-off 1.5 Mb)	Total ROH length (cut-off 1.5 Mb)	Homozygous ratio
Father	44	123,049,309	1.05
Mother	55	251,514,461	2.15
Son	72	413,645,034	3.53
Proband	47	237,564,469	2.03

Table 3. Number and total length of ROHs of the Family 2.

Sample	Number of ROHs (cut-off 1.5 Mb)	Total ROH length (cut-off 1.5 Mb)	Homozygous ratio
Father	31	82,570,772	0.70
Mother	29	65,961,650	0.56
Son	60	368,429,799	3.15
Proband	51	244,287,314	2.09

Supplementary Table S4: CEU populations of the 1000 Genomes Project.		
CEU sample	#ROH regions (cut-off 1.5 Mb)	Total ROH length (cut-off 1.5 Mb)
NA07347	39	106,376,199
NA11831	48	136,881,072
NA11830	38	101,034,986
NA10851	47	129,703,972
NA11832	44	107,741,456
NA07357	55	152,663,835
NA11843	55	149,934,599
NA06989	48	113,842,564
NA07056	53	165,109,126
NA06984	40	122,85,835
NA07037	33	81,158,923
NA06985	48	122,109,441
NA07051	40	91,727,641
NA07048	33	93,706,363
NA11840	40	98,139,215
NA11829	42	129,474,178
NA10847	36	99,052,682

NA07000	48	134,365,628
NA06986	40	114,937,185
NA06994	33	86,687,035