Original Article

Genetic testing in four Indian families with suspected Stickler syndrome

Suganya Kandeeban, Kaustubh Kandale[1], Porkodi Periyasamy, Muna Bhende[1], Pramod Bhende[1], Mathavan Sinnakaruppan, Sripriya Sarangapani[1]

Purpose: Stickler syndrome is associated with the development of rhegmatogenous retinal detachment (RRD), and often presents with ocular, auditory, skeletal, and orofacial abnormalities. Molecular analysis has proven effective in diagnosis, confirmation and classification of the disease. We aimed to describe the utility of next-generation sequencing (NGS) in genetic analysis of four Indian families with suspected Stickler syndrome. **Methods:** The index cases presented with retinal detachment with family history. Genetic analysis in the index case was performed by next-generation sequencing of inherited retinal degeneration genes, and validated by Sanger sequencing followed by co-segregation analysis in the other family members. **Results:** Twenty patients were included for the genetic analysis (15 males and 5 females from four families). Clinical details were available for 15 patients (30 eyes). Fourteen eyes (11 patients) developed RRD. In the 16 eyes without RRD, 8 underwent barrage laser to lattice degeneration and 8 were under observation. Disease segregating heterozygous mutations with pathogenic/likely pathogenic effect was identified in *COL2A1* (c.4318-1G>A, c.141G>A, c.1221+1G>A for 3 families) and *COL11A1* (c.1737+1 G>A for 1 family) gene. In addition to the mutation in the *COL2A1* gene, a pathogenic heterozygous variant associated with risk for arrhythmogenic right ventricular cardiomyopathy (ARVC) was identified in one member. **Conclusion:** NGS testing confirmed the presence of the causative gene for Stickler syndrome in the index case followed by evaluation of family members and confirmation of genetic and ocular findings. We believe that this may be the first such report of families with RRD from India.

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Stickler syndrome is a hereditary arthro-ophthalmopathy associated with orofacial (clefting condition of hard and soft palate), auditory (conductive or sensorineural hearing loss) and musculoskeletal (spinal defects, chronic back pain, bony joint enlargement, hypermobility, premature osteoarthritis) abnormalities.[1–3] The ocular features include retinal detachment, progressive myopia, vitreous anomalies, congenital cataract, glaucoma, etc. Candidate genes with major role in collagen / connective tissue metabolism, adhesion and signaling are implicated in Stickler syndrome.[4] In this report, we discuss the outcome of genetic testing using next-generation sequencing (NGS)–based approach in four families of Indian origin with multiple members, with history of RRD.

Methods

Clinical evaluation

The index cases were referred for genetic analysis from the vitreo retinal services after a detailed ophthalmic evaluation and clinical documentation that included best corrected visual acuity (BCVA), slit-lamp evaluation of the anterior segment, intraocular pressure (IOP) measurement and indirect ophthalmoscopy for fundus evaluation. Details of all intervention—surgery or conservative—were extracted from the medical record. Systemic features (musculoskeletal, auditory, orofacial) were noted. This study was approved by the Institutional Review Board, and performed in accordance with the Declaration of Helsinki.

Laboratory methods

Pedigree charted and blood sample (8 ml) was collected in acid citrate dextrose (ACD) vacutainer after obtaining informed consent from the patient and family members. Genomic DNA was extracted using NucleoSpin® Blood XL kit (Macherey-Nagel, GmbH, Germany) as per the manufacturer's instructions and assessed for its quality and quantity by standard agarose gel electrophoresis and Thermo Scientific NanoDrop™ 1000 Spectrophotometer respectively.

Genotyping was performed for the index cases in Next-Generation Sequencing (NGS) Illumina platform at Strand life science (The Strand® Clinical Exome Test) and

SNONGC Department of Genetics and Molecular Biology, Vision Research Foundation, Chennai, [1]Shri Bhagwan Mahavir Department of VitroRetinal Services, Medical Research Foundation, Chennai, Tamil Nadu, India

Correspondence to: Dr. Sripriya Sarangapani, PhD, Senior Principal Scientist and Associate Professor, SNONGC Department of Genetics and Molecular Biology, Vision Research Foundation, Sankara Nethralaya, Chennai, Tamil Nadu, India. E-mail: vatsanpriya@gmail.com. Dr Muna Bhende, Shri Bhagwan Mahavir vitreoretinal service, Medical Research Foundation, Chennai, India. E-mail: drmuna@snmail.org

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analyzed using STRAND® NGS v2.5 and StrandOmics v6.1 software (Strand Life Science Pvt Ltd), a clinical genomics interpretation and reporting platform. The various *insilico* predictors for the pathogenicity of the variants include HGMD, ClinVar, dbSNP, gnomAD (v2.1), 1000 Genomes and LOVD databases. SIFT, PolyPhen, Mutation taster, Mutation assessor and Variant effect. Variants were annotated, prioritized, and reported based on ACMG guidelines. The candidate genes analyzed included *COL11A1*, *COL11A2*, *COL2A1*, *COL9A1*, *COL9A2*, and *COL9A3* genes.

Validation, Co. segregation analysis and control screening (100 unrelated healthy controls of Indian origin) was performed by PCR [Table 1] based bidirectional sequencing in ABI 3500 Avant Genetic Analyzer (Applied Biosystems, Foster City, USA). The control population was >40 years of age[5] who had undergone complete ophthalmic evaluation which ruled out known phenotypes for inherited retinal diseases.

Results

Clinical evaluation

Twenty patients (15 males and 5 females) who gave their blood sample for genetic analysis were included from four families. A detailed ophthalmic evaluation was available for 15 patients (30 eyes) harboring the disease segregating mutations. Mean age was 28.47 ± 18.3 years (range 5–64 years, with a median of 22 years). Mean age at presentation with RRD was 22.36 ± 17.43 years ranging from 5 to 58 years (median age of 13 years). Eleven patients (14 eyes) developed RRD. 63.63% (7/11) patients developed RRD before 20 years of age.

Table 2 details the ocular features observed (30 eyes) in the proband and family members (N = 15) who were positive for candidate gene mutations. Fourteen eyes had history of RRD. Presenting complaints were diminution of vision in 13 eyes and floaters in 4 eyes. Peripheral retinal changes were noted in 74% (20/27) of eyes. Significant lesions were circumferential lattice degeneration, radial lattice degeneration, and giant retinal tears, the latter two being findings that suggest the presence of STL2. Membranous vitreous abnormalities, a characteristic finding of STL1, were present in 38.46% (10/26) of eyes. 28% (7/25) eyes had developed cataract at presentation. In the 16 eyes without RRD, 8 underwent barrage laser to lattice degeneration and 8 are under observation.

Surgery details are noted in Table 3. The surgical procedures (11 of 14 eyes) included scleral buckle in two eyes, pars plana vitrectomy in two eyes, scleral buckle followed by vitrectomy in one eye, encircling band with vitrectomy in five eyes, and one eye was operated elsewhere. Anatomical success was achieved in all 11 operated eyes. Visual acuity of logMAR 1 or better was seen in six eyes. In two eyes, the condition was deemed inoperable. One eye with RRD was lost to follow-up. Three eyes presented to us with phthisis. Six eyes were on antiglaucoma medications at last visit. Average duration of follow-up was 9.1 ± 10.7 years.

Genetic analysis

Family 2: [Fig. 1a] A truncating variant (c.4318-1G>A) that lies in the essential splice acceptor site was identified in intron 53 of *COL2A1* gene in the index case. Since there was a strong family history of RRD, blood sample was collected from other family members and screened for the specific mutation (c.4318-1G>A). The variant segregated with the disease in all the affected individuals [Fig. 1a]. *In silico* analysis using Human Splice finder suggests that this variant might affect splicing due to disruption in the splice site, resulting in loss of function [Fig. 1e and f].

Family 2: [Fig. 1b] The pedigree showed an autosomal dominant pattern with similar history in father and sibling. The index case was heterozygous for the variation, c.1737 +1 G>A in intron 16 of *COL11A1* gene that segregated in the affected sibling and father. The variant (c.1737+1G>A) lies in the essential splice donor site, predicted to affect splicing due to loss of constitutive splice site [Fig. 1g] and introduction of a new splice site by *in silico* splice prediction tools (ASSP, NNSPLICE, MaxEntScan) and EXSKIP [Fig. 1h], leading to a premature termination of the protein lacking a functional fibrillar collagen NC1 domain.

Family 3: [Fig. 1c]The pedigree showed an autosomal dominant pattern with similar history in mother and sibling. The proband harbored mutations in *COL2A1* and *PKP2* genes. Fig. 2 shows the fundus photographs of the proband and his sibling. Fig. 2 shows the fundus photographs of the proband and his brother. The primary mutation, c.141G>A, p.Trp47Ter, was detected in exon 2 of *COL2A1* gene in the index case and segregated in his sibling. History of high myopia and vision

Table 1: PCR primers and condition validation

Family ID	Gene Name	Exon/Intron	Primer Sequence (5′-3′)	Annealing Temperature (°C)	Amplicon size (bp)
FI	COL2A1	Intron 53	FP: TCAGTTTTGGGCTTCTGGGCA RP: GAGTGACTGAGATTGGAAAGT	55.5	300
FII	COL11A1NM_001854.4	Intron 16	FP: AGTTTCTGAAACCATCTCTCTT RP: GATGTGAATATAAAAATAT	47.6-14 cycles 40.6-19 cycles	365
FIII	COL2A1	Exon 2	FP: GAATTCTGTCAAGTTGAC RP: AAATAAATTACAACCACTGG	51	322
	PKP2 NM_001005242.3	Exon 12	FP: ATGTTCTTCACCCAAAATAT RP: GCTTTACATCCTGTTTGC	52	404
FIV	COL2A1	Intron 19	FP: GAGGTAAGCAGTAGAGGAGGCA RP: TTGGAGCCCACAACTGTC	60	445
	COL11A1	Exon 59	FP: CTGTATATTTCATTCAGAA RP: AAATTAGAATTAAGACAC	48.2-14 cycles 41.2-19 cycles	383

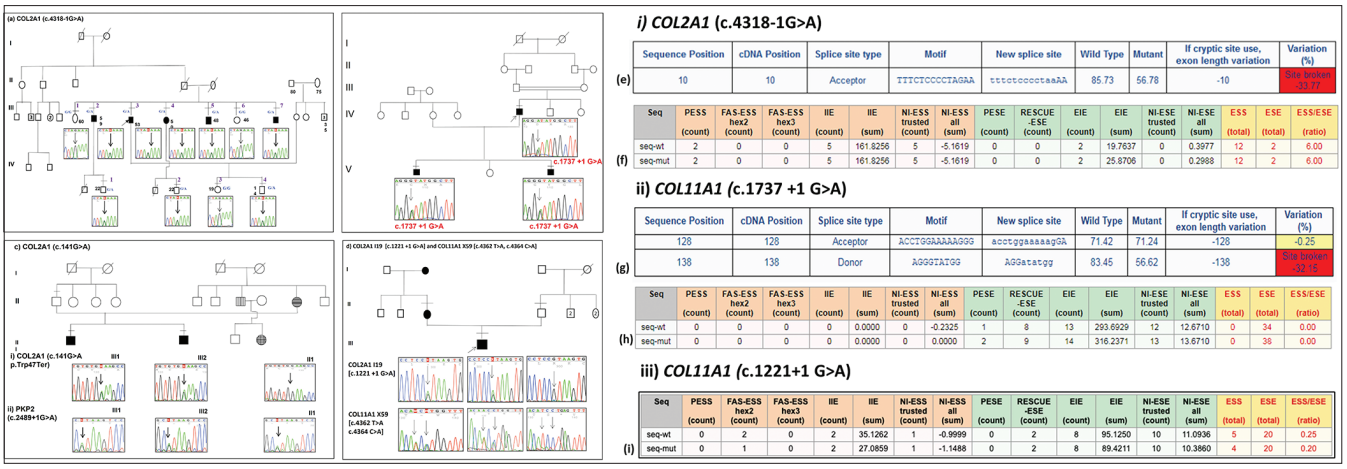
Table 2: The ocular features of the proband and family members (30 eyes) harboring the mutations in candidate genes

Candidate gene	COL2A1			COL11A1	COL2A1 + COL11A1	Total (*n*=30)
Clinical Features	Family 1 (*n*=16)	Family 3 (*n*=4)	Total (*n*=20)	Family 2 (*n*=6)	Family 4 (*n*=4)	
Lens status						
Clear	9	3	12	3	3	18
Mature cataract	5	0	5	0	1	6
Congenital cataract	0	1	1	0	0	1
Pseudophakia	2	0	2	2	0	4
Aphakia	0	0	0	1	0	1
Vitreous						
Clear	6	1	7	2	2	11
Floaters	4	0	4	0	0	4
Vitreous hemorrhage	0	1	1	0	0	1
Vitreous membrane	0	2	2	1	2	5
Vitreous condensation	4	0	4	0	0	4
Silicone oil filled eye	0	0	0	1	0	1
Vitreous Floaters + Vitreous Membrane	1	0	1	0	0	1
No view	1	0	1	2	0	3
Retinal detachment						
No	10	2	12	1	3	16
Yes	6	2	8	5	1	14
Peripheral retinal lesions						
Nil	6	0	6	1	0	7
Circumferential lattice	3	4	7	3	2	12
Radial lattice	3	4	7	1	3	11
GRT	2	1	3	0	0	3
Other retinal breaks	4	3	7	1	1	9
No view	1	0	1	2	0	3
Optic Disc						
Normal	12	4	16	1	3	20
Glaucomatous changes	0	0	0	0	0	0
Myopic	2	0	2	2	0	4
Pale disc	1	0	1	1	1	3
No view	1	0	1	2	0	3

Table 3: Visual acuity and surgical details of the eyes with retinal detachment

	Family 1 (*n*=5)	Family 2 (*n*=3)	Family 3 (*n*=2)	Family 4 (*n*=1)	Total (*n*=11)
No. of eyes with RD	6	5	2	1	14
BCVA in logMAR					
At presentation	1.68±1.06	2.23±1.08	1.9±0.46	2.7	1.94±0.87
At last visit	1.25±1.36	2.23±1.08	0.9±0.46	0.4	1.3±1.12
Surgery details					
No intervention	2	1	0	0	3
Scleral Buckle	0	1	1	0	2
Vitrectomy	0	2	0	0	2
Scleral buckle followed by Vitrectomy	1	0	0	0	1
Encircling band + Vitrectomy	2	1	1	1	5
Operated elsewhere	1	0	0	0	1

loss was noted in proband's maternal uncles (clinical data not available). The affected sibling and unaffected father were heterozygous and wildtype for the variant respectively. This nonsense substitution predicted to cause premature termination of the protein and the truncated protein might have a length of 46 amino acids (aa) as opposed to the original length of 1487 aa.



i) COL2A1 **(c.4318-1G>A)**

(e)

Sequence Position	cDNA Position	Splice site type	Motif	New splice site	Wild Type	Mutant	If cryptic site use, exon length variation	Variation (%)
10	10	Acceptor	TTTCTCCCCTAGAA	tttctccccttaaAA	85.73	56.78	-10	Site broken -33.77

(f)

Seq	PESS (count)	FAS-ESS hex2 (count)	FAS-ESS hex3 (count)	IIE (count)	IIE (sum)	NI-ESS trusted (count)	NI-ESS all (sum)	PESE (count)	RESCUE -ESE (count)	EIE (count)	EIE (sum)	NI-ESE trusted (count)	NI-ESE all (sum)	ESS (total)	ESE (total)	ESS/ESE (ratio)
seq-wt	2	0	0	5	161.8256	5	-5.1619	0	0	2	19.7637	0	0.3977	12	2	6.00
seq-mut	2	0	0	5	161.8256	5	-5.1619	0	0	2	25.8706	0	0.2988	12	2	6.00

ii) COL11A1 (c.1737 +1 G>A)

(g)

Sequence Position	cDNA Position	Splice site type	Motif	New splice site	Wild Type	Mutant	If cryptic site use, exon length variation	Variation (%)
128	128	Acceptor	ACCTGGAAAAAGGG	acctggaaaagGA	71.42	71.24	-128	-0.25
138	138	Donor	AGGGTATGG	AGGatatgg	83.45	56.62	-138	Site broken -32.15

(h)

Seq	PESS (count)	FAS-ESS hex2 (count)	FAS-ESS hex3 (count)	IIE (count)	IIE (sum)	NI-ESS trusted (count)	NI-ESS all (sum)	PESE (count)	RESCUE -ESE (count)	EIE (count)	EIE (sum)	NI-ESE trusted (count)	NI-ESE all (sum)	ESS (total)	ESE (total)	ESS/ESE (ratio)
seq-wt	0	0	0	0	0.0000	0	-0.2325	1	8	13	293.6929	12	12.6710	0	34	0.00
seq-mut	0	0	0	0	0.0000	0	0.0000	2	9	14	316.2371	13	13.6710	0	38	0.00

iii) COL11A1 (c.1221+1 G>A)

(i)

Seq	PESS (count)	FAS-ESS hex2 (count)	FAS-ESS hex3 (count)	IIE (count)	IIE (sum)	NI-ESS trusted (count)	NI-ESS all (sum)	PESE (count)	RESCUE -ESE (count)	EIE (count)	EIE (sum)	NI-ESE trusted (count)	NI-ESE all (sum)	ESS (total)	ESE (total)	ESS/ESE (ratio)
seq-wt	0	2	0	2	35.1262	1	-0.9999	0	2	8	95.1250	10	11.0936	5	20	0.25
seq-mut	0	1	0	2	27.0859	1	-1.1488	0	2	8	89.4211	10	10.3860	4	20	0.20

Figure 1: Co-segregation analysis of (a) family I (FI) *COL2A1* (c.4318-1G>A) (b) family II (FII) *COL11A1* (c.1737 +1 G>A) (c) family III (FIII) *COL2A1* (c.141G>A, p.Trp47Ter) (d) family IV (FIV) *COL2A1* in [c.1221 +1 G>A] and *COL11A1* [c.4362 T>A; c.4364 C>A]. In silico analysis using various splice site prediction tools Human Splice finder (http://www.umd.be/HSF/) (e and g) and EX-SKIP (http://ex-skip.img.cas.cz/) (f, h and i) in genes (i) *COL2A1* (c.4318-1G>A) and *COL11A1* (ii) c.1737 +1 G>A (iii) c.1221+1G>A

The resultant protein lacks a functional fibrillar collagen NC1 domain (residues 1253–1487) causing loss of function. Moreover, due to introduction of a premature stop codon, this aberrant transcript will likely be targeted by the nonsense mediated mRNA decay (NMD) mechanism.[6] The identified variant (c.141G>A) has been reported in the dbSNP database (rs121912896) and ClinVar database, (RCV000018944.27) as a pathogenic variant with respect to Stickler syndrome. In addition, a heterozygous mutation, c.2489+1G>A, was identified in intron 12 the *PKP2* gene as secondary finding. Screening of the family members showed heterozygous genotype in father and sibling. The variant (c.2489+1G>A) lies in the essential splice donor site, in intron 12 of the *PKP2* gene and was predicted to affect constitutive splice site and introduction of a new splice site causing frameshift resulting in premature termination of the protein.

Family 4: [Fig. 1d] The pedigree showed an autosomal dominant pattern with affected mother and maternal grandmother having retinal findings. Fig. 2 shows the fundus photographs of the proband and his mother. The index case showed heterozygous variations in intron 19 (c.1221+1G>A; rs1399741348) of *COL2A1* and exon 59 (c.4362T>A in cis with c.4364C>A) of *COL11A1* genes. The mother was heterozygous and the father showed homozygous wild type genotype for all the three variations. The heterozygous variant (c.1221+1G>A) lies in the essential splice donor site, in intron 19 of the *COL2A1* gene. *In silico* splice prediction tools (ASSP, NNSPLICE and MaxEntScan) suggest that this variant might affect splicing due to the loss of constitutive splice site and introduction of a new splice site [Fig. 1i]. The heterozygous missense substitution in exon 59 of the *COL11A1*gene lies in the collagen-like 7 domain (residues 1429-1487) of the protein gene and alters a conserved residue in the protein. *In silico* analysis (SIFT, LRT, Mutation Taster, Mutation Assessor and FATHMM) predicts a damaging effect for the variants p.His1454Gln and p.Pro1455His.

Discussion

Stickler syndrome often presents with varying orofacial, ocular, auditory, and skeletal abnormalities. There are six distinct molecular types of Stickler syndrome, classified as STL1 to STL6 with mutations in candidate genes involved in collagen synthesis and metabolism. Stickler syndrome differs in their inheritance pattern and classified into STL1 to STL3 (autosomal dominant) and STL4 to STL6 (autosomal recessive).[7] In this report, we have presented a retrospective analysis of four families that were genetically characterized using NGS platform. All the four families presented with autosomal dominant inheritance pattern with co-segregating mutations in COL2A1 and COL11A1 genes [Fig. 1].

Mutations in *COL2A1* gene are reported in 80%–90% of all cases[8] and classified as STL1. Families I and III showed disease segregating heterozygous splice site mutations in *COL2A1* gene with clinical significance of likely pathogenic based on *in silico* predictions, thus confirming the molecular type as STL1 (type II collagenopathy). The reported mutation spectrum in *COL2A1* gene (chromosomal loci 12q13.11) includes prevalent nonsense mutations (68%) and arginine-cysteine substitutions in one third of cases, involving the X position in the G-X-Y repeat. The glycine substitutions represent only 6% of the mutations found in STL1. The types of mutations ranged from substitutions resulting in premature stop codon, cryptic splice sites triggering nonsense mediated decay to entire gene deletions.[9] These mutations alter alpha-1 chains and result in abnormal conformation and destabilization of the triple helix. In this study, we observed splice site mutation in two families (family I and II) and tryptophan substitution in the *COL2A1* gene in one family.

In family III, c.2489+1G>A in *PKP2* gene was identified as secondary mutation in heterozygous state. Germline pathogenic variations in the *PKP2* gene have been shown to be associated with arrhythmogenic right ventricular cardiomyopathy (ARVC).[10,11] History of cardiac stent fixation and eye problem was noted in maternal uncle; further clinical correlation was not possible as the clinical and genetic data were not available for them. There weren't any related complaints in the father who was heterozygous for the same. As the identified variant (rs111517471) with ExAc allele frequency of <0.01% with a ClinVar ID of RCV000157417.4 was associated with ARVC,

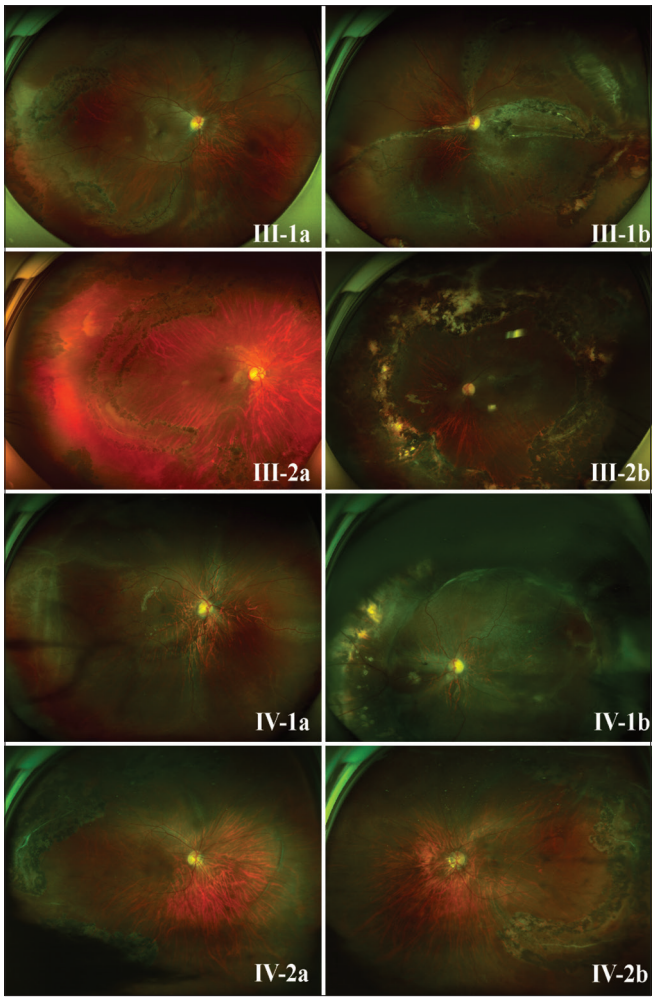


Figure 2: These fundus photographs show two family members each of Family 3 and 4. Picture 31a shows the right eye of the primary case of family 3 which shows radial and circumferential lattices which have been lasered. Picture 31b shows the left eye of the primary case which developed retinal detachment and underwent scleral buckling and barrage laser photocoagulation to the lattices. Picture 32a shows the right eye of the brother of the primary case who also shows radial and circumferential lattices which have been lasered. Picture 32b shows the left eye of the brother of the primary case who developed retinal detachment and underwent vitrectomy. Picture 41a shows the right eye of the primary case which shows radial and circumferential lattices with vitreous membranes. Picture 41b shows the left eye of the primary case of family 4 which developed retinal detachment and underwent vitrectomy. Picture 42a and 42b show the right and left eye respectively of the mother of the primary case who also shows radial and circumferential lattices which have been lasered

the patients and the father were advised relevant medical consultation. The *PKP2* gene encodes the protein, plakophilin-2, that links cadherins to intermediate filaments in the cytoskeleton and may regulate the signalling activity of beta-catenin.

COL11A1 gene mutations: Stickler cases with *COL11A1* gene mutations are classified as type II (STL2) that accounts for 10%–20% of cases. We observed disease segregating splice site mutations (c.1737 +1 G>A) in intron 16 of *COL11A1*gene in family II. The identified variant has not been previously reported in literature. Splice variants, such as c.3204+1G>T (RCV000389916.1) and c.3816+1G>A (RCV000032995.32), lying downstream of the identified variant, have been reported as "pathogenic" in the ClinVar database with respect to Stickler syndrome and Marshall syndrome (a variant form of Stickler syndrome), respectively. Thus, this variant has been labelled as "likely pathogenic".

We observed three genetic variations in family IV that included heterozygous mutations in *COL2A1* (c.1221+1G>A) gene with likely pathogenic classification and two variants of unknown significance (VUS) in exon 59 (c.4362 T>A; c. 4364 C>A) of *COL11A1* gene. *COL2A1* gene variant (c.1221+1G>A) has been previously reported (rs1399741348) in Stickler syndrome patient.[12] The variant is likely to disrupt an essential splice donor site, which might result in loss-of-function; however, further experimental evidences are needed to prove the exact disease association.

COL2A1 gene mutations (with a major role in regulating collagen fibrillogenesis) were reported in association with congenital membranous vitreous anomaly[7] and correlated with beaded bundles of irregular diameters in the vitreous gel. Phenotype and genotype correlation of a large series of Stickler syndrome patients with *COL2A1* gene mutation has shown strong correlation with retinal tears or detachments, cleft palate and a positive family history, and these features were concluded as a good indicator of STL1.[12] The index case of family IV was positive for all these features thus favoring the molecular diagnosis of STL1.

COL11A1 mutations were associated with vitreoretinal anomalies and sensorineural hearing loss in Stickler syndrome patients[13]; in our study, we did not record similar history in family IV harboring *COL11A1* gene mutations. Screening of controls are however required to prove the pathogenicity status of the observed VUS in *COL11A1* gene and to confirm the molecular classification.

A recent review on the mutation spectrum of STL1 genes and phenotype correlation in East Asian population showed that patients with splicing mutations developed cataract and had severer systemic phenotype like arthropathy when compared to patients with truncating mutations.[14] A similar observation was made in all the three families (FI, FIII and FIV) that harbored splice site mutations in *COL2A1* gene.

Retinal detachment and vitreous changes were observed in all the patients irrespective of the genetic etiology. Radial lattice degeneration and giant retinal tears were earlier associated with Stickler syndrome. While history of RD was present in all four families irrespective of the genetic etiology, associated cleft palate as a systemic feature was observed in family IV. Myopia was observed in two families. No case showed specific optic disc changes [Table 2]. The average age at presentation with retinal detachment was similar to the study by Wang *et al*.[14] but was less than that reported by Liberfarb *et al*.[15] All the cases in this study were myopic as described in previous literature.[2,16,17] Association of cataract was as that reported by Wang *et al*.[14]

In the present study, we confirmed the molecular type (family I and III as STL1 and family II as STL2) and all of them exhibited autosomal dominant inheritance. The molecular classification for family IV was more towards STL1 based on the suggested clinical indicators correlated with *COL2A1* gene; however, this could be confirmed after analyzing the status of

VUS observed in *COL11A1* gene. An additional mutation in *PKP2* gene (associated with ARVC) was identified in family III but patients were asymptomatic for the same at the time of genetic diagnosis. They were advised for relevant medical screening and management. Genetic testing using the clinical exome panel was thus helpful in predicting the risk for cardiomyopathy in this family. To the best of our knowledge, this is the first series of Indian families with RRD where results of genetic testing for Stickler syndrome has been reported.

Conclusion

Next-generation sequencing confirmed Stickler syndrome in the index case of each of the four families followed by evaluation of family members and confirmation of genetic and ocular findings**.** Molecular screening in Stickler syndrome patients could prove useful for diagnosis, confirmation, and classification. Our data suggests that clinical exome analysis proves beneficial in predicting the risk of associated systemic features in asymptomatic individuals. A combined clinical and molecular genetic analysis thus helps in distinguishing ocular and non-ocular variants which delineates the need for further investigation or clinical follow-up in these patients for better disease management and potential counselling benefits.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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