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WHOLE EXOME SEQUENCING IDENTIFIED A PATHOGENIC MUTATION OF *COL2A1* CAUSING STICKLER SYNDROME IN A VIETNAMESE FAMILY

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SUMMARY

Stickler syndrome is a group of rare inherited diseases associated with abnormalities in connective tissues, specifically collagen of the eyes, ears, craniofacies, skeleton and joints. The inheritance pattern of this disease is either an autosomal dominant or an autosomal recessive based on the causative gene. Stickler syndrome is characterized by severe nearsightedness, vitreous abnormalities, distinctive facial features, hearing problems and joint anomalies. Herein, we report a case of a 37-year-old man from Vietnam suspected of Stickler syndrome, presenting a phenotype of retinal detachment and complete loss of vision, and his 3-year-old son with congenital high myopia and vitreous abnormalities. Genetic analysis using whole exome sequencing (WES) revealed a nucleotide substitution (c.C2818T/p.R940X) in exon 42 of the *COL2A1* gene that was previously reported as a pathogenic variant causing Stickler syndrome. Validation of *COL2A1* c.C2818T in all members of this family by using Sanger sequencing detected the presence of this pathogenic variant in the heterozygous form in the affected father and son but not in the mother and another son without any signs of a vision problem. Thus, our study contributes to not only the knowledge base of clinical and genetic aspects of Stickler syndrome in Vietnam but also the awareness of the importance of genetic counseling in patients with *COL2A1* c.C2818T mutation, as well as early diagnosis and appropriate treatment to prevent serious complications, especially blindness.

Keywords: *Whole Exome Sequencing (WES), Stickler syndrome, COL2A1 gene, congenital high myopia, retinal detachment*

INTRODUCTION

Stickler syndrome is a group of rare disorders related to abnormalities in connective tissues, specifically collagen (Stickler *et al.*, 1965). Gunnar Stickler and his colleagues firstly studied and described this group of disorders as the disorder hereditary progressive arthro-ophthalmopathy. It often exerts effects on the connective tissue of the eyes, ears, craniofacies, skeleton and joints (Robin *et al.*, 1993). People with Stickler syndrome experience a wide variety of signs and symptoms that typically differ among individuals (Stickler *et al.*, 2001). One of the first signs of this syndrome is nearsightedness (myopia). The majority of patients suffered from myopia that was usually severe, congenital and non-progressive (Snead, Yates, 1999). Furthermore, vitreous abnormalities are also serious conditions in

Stickler syndrome divided into two phenotypes (Snead, Yates, 1999). Type 1 phenotype found in most of patients is characterized by vestigial vitreous gel locating in immediate retrolental space and bordered by a folded membrane. Type 2 phenotype presents in a minority of subjects and is characterized by distinct bundles throughout the vitreous cavity that are sparse and irregularly thickened. These eye problems could lead to either impaired vision or significant blindness. Distinctive facial features include a flattened facial appearance and Pierre Robin sequence including cleft palate, glossoptosis and micrognathia. Additionally, hearing loss, mitral valve prolapse and musculoskeletal abnormalities associated with joints and bones can also occur.

According to National Organization for Rare Disorders (Accessed November 27th, 2020), Stickler syndrome has been divided into six types. Stickler syndrome type I, II and III, respectively caused by mutations of *COL2A1* gene on chromosome 12q13.11, *COL11A1* gene on chromosome 1p21 and *COL11A2* gene on chromosome 6p21.3, are inherited in an autosomal dominant pattern. An affected person can either receive one mutation allele from one affected parent or have a spontaneous genetic change occurring during formation of gametes or embryonic development (a de novo mutation). These genes are responsible for the production of type II and type XI collagen. Stickler syndrome type I which leads to loss-of-function *COL2A1* gene accounts for 80 to 90% of all reported cases while type II represents 10 to 20% of cases (Robin et al., 1993). Type III is described as a non-ocular form of Stickler syndrome because it has no impact on eyes. Stickler syndrome type IV, V and VI, in turn, occur due to mutations of *COL9A1* gene on chromosome 6q13, *COL9A2* gene on chromosome 1p33 and *COL9A3* gene on chromosome 20q13. The inheritance pattern of these three types is autosomal recessive. Mutations in any genes associated with Stickler syndrome would lead to defective collagen molecules or insufficient amount of collagen, resulting in the impairment of connective tissue of the body. It was estimated that Stickler syndrome affects an approximate incidence of 1:7,500-9,000 newborns (Robin *et al.,* 1993).

The purpose of this study is to determine the causative mutation of a father and his son with suspected Stickler syndrome based on ocular signs characteristics. We performed whole exome sequencing analysis for the affected father and searched for variants on the six genes known to have relevance for Stickler syndrome. The pathogenic or likely pathogenic variant was then checked in the proband's family members by using Sanger sequencing. The correlation between identified pathogenic variants and the related clinical manifestations was also discussed in our study.

MATERIALS AND METHODS

Subjects and genomic DNA extraction

The subjects of this study was a father (I.1) and his first son (II.1) with ophthalmologic manifestations of Stickler syndrome. He had no family history of ocular disease while his wife (I.2) and the second son (II.2) are normal in vision. The proband were examined and monitored by ophthalmologists in Vietnam National Hospital of Ophthalmology in Hanoi. The request for genetic testing for Stickler syndrome risk was given by ophthalmologist to confirm the diagnosis. This study was carried out with the agreement of the patient's family and was approved by the Ethics Committee in Biomedical Research of the Institute of Genome Research.

Peripheral blood samples were obtained from patient and family members, genomic DNA subsequently was extracted from leukocytes using standard protocols. Concentration of total DNA was then determined by Qubit dsDNA BR Assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Whole exome sequencing

DNA genome isolated from proband I.1 was applied for whole exome sequencing.

Library constructions was performed by Sure Select V6-Post using manufactural protocol (Agilent Technologies, Santa Clara, California, USA). The sequencing library is prepared by random fragmentation of DNA, following by 5' and 3' adapter ligation. Fragments ligated with adapter were subsequently amplified by PCR and gel purified. Enriched library was quantified using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). DNA fragments distribution was confirmed by 2100 Bioanalyzers using High sensitivity DNA chip (Agilent Technologies, Santa Clara, California, USA) with expected size range from 200 bp to 400 bp. Paired-end sequencing was carried on the NovaSeq platform (Illumina, San Diego, California, USA) following the manufacturer's instructions. The mean exome coverage was more than 100X and each target base having at least 20X coverage.

Sanger sequencing

Direct Sanger sequencing method was used to validate the candidates of pathogenic variants detected in the proband (I.1) by WES as well as in their familiar members. Primers provided by PHUSA Biochem Company (Can Tho, Vietnam) with a forward specific primer 5′-AGGTAGAGAAGCCCCCAGAG-3′ and a reverse primer 5′-TGAGAGAGGAGAGGCAGGAG -3′, respectively. PCR reaction was performed with a total volume of 20 µL containing: 10 ng total genomic DNA, 1X NEB Master mix (New England Biolabs, Ipswich, Massachusetts, USA), 10 pmole of each primer, 1 µL DMSO (Bioworld, Dublin, Ohio (OH) 43017, USA) and 16.5 µL deionized water. The thermo cycle was as following: denaturation at 95°C for 5 min, following by 40 cycles of 95°C for 15 sec, 58°C for 30 sec, 68°C for 20 sec and a final extension at 68°C for 5 min. PCR products were then purified by Multiscreen PCR 96 Filter Plate (Merck-Millipore, Burlington, Massachusetts, USA) and subsequently bi-direction sequenced using ABI Prism BigDye Terminator Cycle Sequencing Kit Version 3.1 (Applied BioSystem, Waltham, Massachusetts, USA), on an ABI genetic analyzer 3500 (Applied Biosystems, Waltham, Massachusetts, USA).

Data analysis

BWA (ver 0.7.10) was used to align raw sequence to the UCSC human reference genome hg19. SNPs and Indels were detected by GATK (ver 3.6) and SAMtools variant caller (ver 1.8). Removing duplicate sequence reads was performed by Markduplicates in the Picard sorfware package. Several DNA databases consisting of 1000 Genome, dbSNPs and gnomAD (ver 2.2.1 https://gnomad.broadinstitute.org/) were used in order to filter out the earlier reported common variants with frequency above 1% as well as synonymous substitutions.

RESULTS

Clinical characteristics of family members

Two patients, who are father and son, were hospitalized for a routine eye examination in February 2020. The father (I.1), 37-year-olds, had high myopia since childhood and then was diagnosed with retinal detachment in both eyes at the age of 20. He underwent scleral buckle surgery and vitrectomy. In 2009, because of retinal re-detachment and 2nd glaucoma and severe pain, both eyes had been eviscerated. His parent and siblings had no symptom of eye disease. The son (II.1), three-year-olds, was diagnosed with congenital high myopia in October 2018. He was full-term and his mother's pregnancy was normal. Refraction errors with cyclopentolate 1% was -8.00/-1,50x180 in the right eye and -10.00/-1,00x180 in the left eye. Best corrected visual acuity (BCVA) of the right eye was 20/100 and the left eye was 20/400. Intraocular pressure was normal. The anterior segment of both eyes was normal. Vitreous floaters were observed, particularly, the left vitreous was more opacity than the right eye. Lattice degeneration was observed at the periphery of the right eye while retinal detachment inferior over the macular was recorded at the left eye. Other members of this

family were normal with no ocular problems. Treatment: right eye wearing glass: Oct 2020 BCVA 20/30. Left eye: scleral buckling, vitrectomy and lensectomy- Oct 2020: VA CF 2m, retinal reattached.

Other examinations revealed that none of these patients displayed anomalies in craniofacies, joints and hearing ability.

Identifying variants in the genes associated with the Stickler syndrome

Genetic variants of six genes including *COL2A1, COL9A1, COL9A2, COL9A3, COL11A1* and *COL11A2* were extracted from WES data of the proband (I.1) and summarized in the Table 1. The total number of variants were 14, including 9 synonymous, 3 non-synonymous and 2 stop-gain variants. Twelve of them were evaluated to be benign or likely benign according to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/). One synonymous variant (*COL11A1* c.C2104A) that has not been reported in all databases is likely benign because it causes no change in amino acid sequence. The most notably variant c.C2818T (p.R940X) locating in exon 42 of the *COL2A1* gene was the only one reported as pathogenic mutation in the ClinVar database. This variant results in a substitution of a codon for arginine residue for a stop codon that signals the end of translation. According to the interpretation in ClinVar database, this genetic disorder was predicted to cause loss-of-function protein by protein shortening or nonsense-mediated mRNA decay that removes transcripts of premature stop codon-containing mRNA. Thus, *COL2A1* c.C2818T mutation was considered to be a causative mutation of Stickler syndrome in the proband of our study. In the dbSNP and ClinVar databases, *COL2A1* c.C2818T was reported at the SNP ID rs1057524114. The mutation was first identified by Kondo and others in Japanese patients with Stickler syndrome (Kondo et al., 2016). None of variant was detected in the *COL11A2* gene.

Table 1. Mutations of six genes associated with Stickler syndrome of the proband.

Gene	Exon	Nucleotide change	Amino acid change	Reference SNP ID	Hom/ Het	Clinical significance
COL2A1 (NM_001844)	1	c.A25T	p.T9S	rs3803183	hom	Benign/Likely_benign
	7	c.C504A	p.G168G	rs3737548	het	Benign/Likely_benign
	34	c.C2295T	p.G765G	rs2276454	hom	Benign/Likely_benign
	36	c.T2400C	p.N800N	rs1635553	hom	Benign/Likely_benign
	40	c.C2673G	p.G891G	rs41272029	het	Benign/Likely_benign
	42	c.C2818T	p.R940X	rs1057524114	het	Pathogenic
COL9A1 (NM_001851)	11	c.T1015C	p.S339P	rs592121	het	Benign
	35	c.G2271A	p.P757P	rs2072650	het	Benign/Likely_benign
COL9A2 (NM_001852)	19	c.A977G	p.Q326R	rs2228564	het	Benign
	19	c.C976T	p.Q326X	rs12077871	het	Benign/Likely_benign
COL9A3 (NM_001853)	2	c.C93A	p.P31P	rs2273078	het	Benign/Likely_benign
	2	c.C129T	p.P43P	rs2273079	het	Benign
	30	c.T1740C	p.P580P	rs2294995	het	Benign
COL11A1 (NM_080630)	28	c.C2104A	p.R702R	Novel	het	Unknown

Hom: homozygous; het: heterozygous.

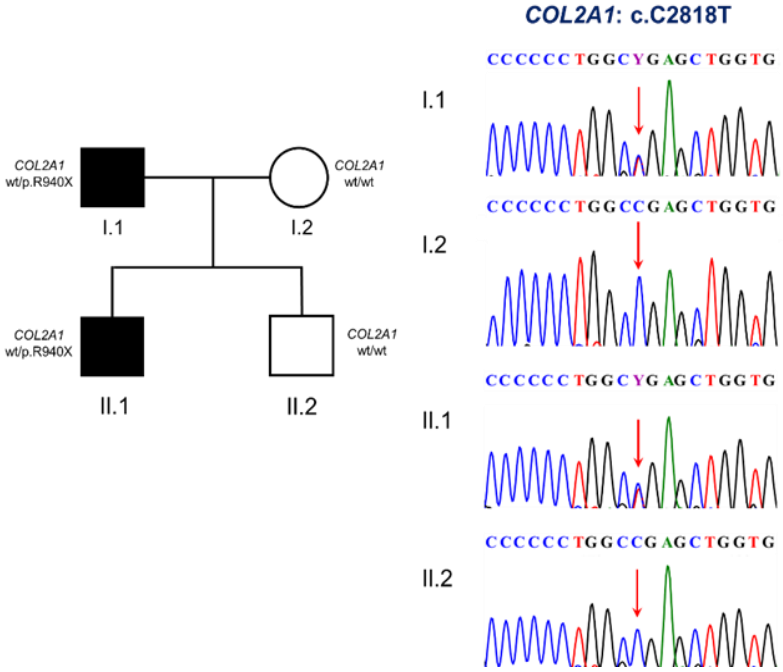

Figure 1. Pedigree of the family and sequencing chromatograms illustrating the substitution of nucleotide C to T (red arrow) at position 2818 of the coding sequence of the *COL2A1* gene.

Examining of the *COL2A1* c.C2818T (p.R940X) mutation in proband family

After identifying the pathogenic mutation c.C2818T in *COL2A1* gene of the proband, the direct sequencing was applied to find whether this mutation was presented in other family members. The results showed that both the affected father and son have the heterozygous c.C2818T mutation in *COL2A1*, while the healthy mother and son have two copies of normal allele. The heterozygous mutation state was completely correlated with dominant pattern of Stickler syndrome caused by *COL2A1* mutation.

DISCUSSION

Type I Stickler syndrome arising from mutant *COL2A1* gene is characterised as an inherited, autosomal dominant disorder. Snead and Yates highlighted that the majority of patients with Stickler disease caused by *COL2A1* gene have congenital, non-progressive and severe nearsightedness, and high risk of retinal detachment (Snead, Yates, 1999). A survey conducted by Stickler and others also showed a 95% of subjects having eye problems including retinal detachment, myopia and blindness (Stickler *et al.*, 2001). Retinal detachment is a very dangerous ophthalmic complication associated with a risk of blindness. In this family, identification of severe nearsightedness and retinal detachment in two affected members was made prior to molecular-genetic analysis. It is irrefutable that prevention is better than cure, thus early detection of Stickler syndrome plays a key role in visual outcome and prevention of blindness. As both the father and affected son carry *COL2A1* c.C2818T mutation in heterozygous form that is inherited in an autosomal dominant manner, their offspring would have a 50% chance of receiving this pathogenic variant and suffering from Stickler syndrome. In consequence, the prenatal testing is required so that the early intervention and surveillance can effectively treat and control the condition, accordingly, protect patients from dangerous complications, specifically blindness.

The prevention of blindness in Stickler syndrome, in addition, has been upgraded through managing four processes: early diagnosing Stickler disease, early identifying retinal detachment, preventing giant retinal tear and treating giant retinal tear detachment (Shapiro *et al.*, 2018).

COL2A1 encodes a constituent subunit of procollagen molecules named pro-alpha1 (II) chain which is processed by enzymes to form mature type II collagen. Type II collagen is a fibrillar collagen providing structure and strength to connective tissues in cartilage, vitreous, inner ear and nucleus pulposus (National Center for Biotechnology Information, Accessed November 16th, 2020). Heterozygous mutations of *COL2A1* underlie several disorders collectively termed type II collagenopathies (Spranger et al., 1994). As listed in the Leiden Open Variation Database (Accessed November 27th, 2020), the total number of *COL2A1* mutations identified is 1132 (updated on October 01, 2020). Notably, almost 200 *COL2A1* variants have been reported to cause the major type of Stickler syndrome classified as type I, according to the Medline Plus (Accessed November 27th, 2020). Most of mutant *COL2A1*-carrying patients have a premature stop signal leading to haploinsufficiency, followed by the underproduction of type II collagen. Similarly, the *COL2A1* c.C2818T variant found in this family is also a nonsense mutation due to the appearance of a stop codon at position 940 of protein. This is the first-time clinical features of type I Stickler syndrome caused by *COL2A1* c.C2818T variant have been reported in Vietnam.

The signs and symptoms of type I Stickler Syndrome resulting from mutant *COL2A1* gene encompass type 1 congenital vitreous abnormalities, retinal detachment, precocious osteoarthritis, normal hearing or mild sensorineural hearing loss and facial abnormalities (Robin et al., 1993). Type II Collagen is the predominant components in cartilage (Gelse et al., 2003). However, in our study case, only ocular problems were observed, whereas no aberrant expression was detected in other features including cartilaginous structures. Similarly, a study about another *COL2A1* variant conducted by Ahmad and his colleagues also suggested that the insufficiency of type II Collagen exerts more effects on eyes, rather than cartilage (Ahmad *et al.*, 1991).

In conclusion, our study reported a case of *COL2A1* c.C2818T mutation as a pathogenic variant causing Stickler syndrome in two members of a Vietnamese family. This finding provides useful information to the knowledge of clinical and genetic basis of Stickler syndrome in Vietnam, and additionally reinforces the importance of genetic counselling in patients with mutant *COL2A1* gene, as well as early diagnosis and appropriate treatment to prevent serious complications, especially blindness.

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GIẢI TRÌNH TỰ HỆ GEN MÃ HÓA XÁC ĐỊNH ĐỘT BIẾN GEN *COL2A1* GÂY HỘI CHỨNG STICKLER Ở MỘT GIA ĐÌNH NGƯỜI VIỆT NAM

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TÓM TẮT

Hội chứng Stickler là một nhóm bệnh di truyền hiếm liên quan đến những bất thường trong mô liên kết, cụ thể là collagen ở mắt, tai, vùng sọ mặt, xương và khớp. Hội chứng này di truyền trên nhiễm sắc thể thường, có thể trội hoặc lặn tùy theo loại gen gây bệnh. Hội chứng Stickler được đặc trưng bởi cận thị nặng, thủy tinh thể dị thường, các đặc điểm khuôn mặt đặc biệt, các vấn đề về thính giác và dị tật khớp. Trong nghiên cứu này, chúng tôi báo cáo một trường hợp về một người đàn ông 37 tuổi ở Việt Nam mắc hội chứng Stickler bị bong võng mạc và mất thị lực hoàn toàn, và cậu con trai 3 tuổi bị cận thị bẩm sinh và dị tật thủy tinh thể. Phân tích di truyền bằng cách sử dụng giải trình tự toàn bộ vũng mã hóa (WES) phát hiện một thay thế nucleotide trong exon 42 của gen *COL2A1* (c.C2818T/p.R940X). Biến thể này đã được báo cáo trước đây là một biến thể gây ra hội chứng Stickler. Kiểm tra đột biến *COL2A1* c.C2818T ở tất cả các thành viên của gia đình này bằng phương pháp giải trình tự Sanger đã phát hiện biến thể này có ở dạng dị hợp tử ở cha và người con trai bị bệnh nhung không có ở người mẹ và người con trai còn lại khỏe mạnh. Nghiên cứu của chúng tôi không chỉ đóng góp vào nền tảng kiến thức về các khía cạnh lâm sàng và di truyền của hội chứng Stickler ở Việt Nam, mà còn nâng cao nhận thức về tầm quan trọng của việc tư vấn di truyền ở bệnh nhân có đột biến *COL2A1* c.C2818T, cũng như việc chẩn đoán sớm và điều trị thích hợp giúp ngăn ngừa các biến chứng nghiêm trọng, thậm chí là mất thị lực hoàn toàn.

Từ khóa: *Giải trình tự hệ gen mã hóa (WES), hội chứng Stickler, gen COL2A1, cận thị nặng bẩm sinh, bong võng mạc*