ORIGINAL ARTICLE

Genetic Heterogeneity of β-thalassemia Variants in Affected Filipinos

Catherine Lynn T. Silao, MD, PhD,[1,2]* Ma. Carmina C. Manuel, PhD,[3] Ronnette Anne E. Davila,[1]
Terence Diane F. Fabella, MS,[1] Rita P. Laude, PhD,[3] Thomas Gabriel H. Desengaño,[1] Carl Angelo S. Estrada,[1]
Mayceemae M. Barnuevo, MPH,[1] Josept Mari S. Poblete, MD, PhD,[2] Carmencita D. Padilla, MD, MAHPS,[1,2]
Maria Liza T. Naranjo, MD[4] and Ernesto dJ. Yuson, MD[5]†

[1]*Institute of Human Genetics, National Institutes of Health, University of the Philippines Manila, Manila, Philippines*
[2]*College of Medicine and Philippine General Hospital, University of the Philippines Manila, Manila, Philippines*
[3]*Institute of Biological Sciences, Genetics and Molecular Biology Division, University of the Philippines Los Baños, Laguna, Philippines*
[4]*National Children's Hospital, Quezon City, Philippines*
[5]*Lung Center of the Philippines, Quezon City, Philippines*

ABSTRACT

Background. β-thalassemia, a hereditary blood disorder, is characterized by the reduction or absence of β-globin chain synthesis, with disease severity depending on population-specific HBB variants. This is the first report to present the molecular profile of Filipino β-thalassemia patients.

Methods. Deletion analysis, β-globin StripAssay analysis, and HBB gene sequencing were performed in 237 patients with β-thalassemia.

Results. Twenty-one (21) previously published β-thalassemia variants – ten (10) pathogenic point mutations [c.79G>A (HbE) (48.52%), c.92+2T>C (1.47%), c.52A>T (0.84%), c.92+5G>C (0.63%), c.-50A>C (cap+1) (0.42%), c.79G>T (0.21%), c.59A>G (Malay) (0.21%), c.47G>A (0.21%), c.20A>T (HbS) (0.21%), and c.2T>G (initiation codon) (0.21%)]; five (5) pathogenic deletions [*HBB* Filipino deletion (23.42%), c.203_204delTG (5.49%), c.126_129delCTTT (codon 41/42) (1.68%), c.155delC (0.63%), and c.110delC (0.21%)]; two (2) conflicting patterns of pathogenicity [c.27dup (0.42%) and c.170G>A Hb J-Bangkok (0.21%)]; and four (4) polymorphisms [c.9T>C (codon 2) (40.93%), c.315+16G>C (36.92%), c.316-185C>T (25.74%), and c.-92C>G (0.21%) – were identified.

Conclusion. The genetic heterogeneity of β-thalassemia in affected Filipinos emphasizes the value of mutation-based confirmation for diagnosis, carrier screening, genetic counseling, disease management, and formulation of nationwide health policies.

Keywords: beta thalassemia, Filipino, HBB, molecular, variant

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Corresponding author: Catherine Lynn T. Silao, MD, PhD
Institute of Human Genetics
National Institutes of Health
University of the Philippines Manila,
623 Pedro Gil St., Ermita, Manila 1000, Philippines
Email: ctsilao@up.edu.ph

INTRODUCTION

Thalassemia, a group of autosomal recessive hereditary hemoglobinopathies, is caused by inefficient or non-synthesis of normal globin chains, leading to ineffective erythropoiesis, microcytosis, and anemia. The most common forms, alpha- and beta-thalassemia, result from mutations in the alpha- and beta-globin gene clusters (*HBA1*, *HBA2*, and *HBB*) located on chromosome 16 and chromosome 11, respectively.[1]

Beta-thalassemia (β-thalassemia), a result of population-specific pathogenic variants in the *HBB* gene, is characterized by decreased hemoglobin in red blood cells (RBC), and consequently, microcytic, hypochromic anemia with varying degrees of severity. It is further classified into β-thalassemia minor (β^+/β or β^0/β), β-thalassemia intermedia (β^+/β^+; β^+/β^0), and β-thalassemia major (β^0/β^0), depending on the presence and zygosity of *HBB* gene variants. Clinical manifestations of β-thalassemia minor range from asymptomatic to mild symptoms; β-thalassemia intermedia may not express severe complications, while β-thalassemia major may cause life-threatening anemia resulting in the need for blood transfusions. Commonly reported clinical profiles of suspected beta-thalassemia patients start at HbA2 level >3.5, normal or elevated RDW values, increased red blood cell (RBC) and anemia levels, decreased hematocrit, and a mean cell volume (MCV) less than or equal to 76 fl.[2] Iron overload, hepatosplenomegaly, and osteoporosis are among its common disease-related complications as a result of transfusion therapy.[3] It can also be associated with other structural hemoglobin variants such as sickle cell (HbS) and hemoglobin E (HbE), which are considered the most observed single-gene disorders worldwide.[4]

This hemoglobinopathy is highly widespread in the malaria-prone areas of Africa, the Mediterranean region, the Middle East, the Indian subcontinent, and Southeast Asia, with population migration and intermarriage now making β-thalassemia a common disease worldwide.[5] Unfortunately, this hematological disorder imposes considerable financial burdens on families and society, thereby making it a crucial health concern. In fact, β-thalassemia has an incidence of roughly 25,000 infants born annually, with a high prevalence of carriers comprising 1.5% of the global population (80–90 million people), while the total annual incidence of symptomatic individuals globally is estimated at 1 in 100,000.[2,6] In the Philippines, the 2024 prevalence of β-thalassemia among screened newborns is recorded at 1:145,248.[7]

Currently, novel thalassemia treatments involving gene editing and regulation of ineffective erythropoiesis that address receptor and signaling pathways are being developed. Treatment options such as blood transfusion and iron chelation, stem cell transplantation, and gene therapies are associated with several challenges and limitations, resulting in difficulties in compliance, thereby affecting prognosis.[8] Comprehensive national prevention programs, including public education and awareness, carrier screening, genetic counseling, prenatal diagnosis, and preimplantation diagnosis have been successful in several countries. However, this is not always true for low and middle-income nations such as the Philippines. Prevention and control of thalassemia by studying the carrier rate and its molecular characterization in different populations is thus necessary.[9,10]

Several molecular studies have reported the heterogeneity of β-thalassemia with significant geographical differences and a large number of population-specific variants that are mostly due to single-nucleotide substitutions in functionally important regions and to insertions-deletions leading to frameshifts.[11,12] At present, there are more than 1,156 beta thalassemia variants described.[13,14] To date, there have been no reports on the molecular profile of this disease in the Philippines, and this is the first study to investigate the molecular defects in highly suspected Filipino patients with β-thalassemia in the country.

METHODS

Study Participants

The study was conducted on 237 unrelated, non-consanguineous Filipino adult patients (n=85) highly suspected of β-thalassemia and newborns (n=152) screened positive for β-thalassemia through the Expanded Newborn Screening Program from 2019 to 2021. All participants underwent pre-genetic counseling and consented to participate in the study.

The diagnosis of β-thalassemia relied on the mean corpuscular volume (MCV) of <79.0 fL, mean corpuscular hemoglobin (MCH) of <27.0 pg, and/or increased fetal hemoglobin (HbF) of >1% and hemoglobin A2 (HbA2) levels of around 3.5-5.0%.[15] Qualitative and quantitative determination of hemoglobin components and levels in screened newborn patients was performed using the VARIANT nbs high-performance liquid chromatography (HPLC) system (Bio-Rad Laboratories, Inc.; CA, USA).

This study adhered to the principles in the Declaration of Helsinki and was conducted with the approval of the University of the Philippines Manila Research Ethics Board (UPMREB, 2014 122 01).

Molecular Analysis

According to the manufacturer's instructions, genomic DNA (gDNA) was isolated from four (4) mL peripheral blood samples (QIAamp DNA Blood MIDI Extraction Kit, Qiagen, Hilden, Germany) and was subsequently screened for β-thalassemia variants via deletion analysis, β-Globin StripAssay SEA™ analysis, and direct sequencing of the *HBB* gene. These tests were performed to cover all three exons of the *HBB* gene to detect single-nucleotide variants and deletions.

Deletion Analysis

Analysis for the 45-kb Filipino (FIL) deletion was performed via gap-PCR in a 10 µL PCR mixture consisting of 30-50 ng genomic DNA, 10x PCR buffer, 50 mM $MgCl_2$, 10 mM dNTPs, 10 µM forward and reverse primers, and 5 U *Taq* polymerase.[16] PCR amplifications were done using a Cycloud A300 Fast Gradient Thermal Cycler (Hangzhou LongGene Scientific Instruments Co., Ltd., China). After initial heating at 94°C/5m, the reaction was followed by 30 cycles at 94°C/30s, 58°C/30s, 72°C/1m, and a final extension at 72°C/5m. The amplified fragments were then separated using 2% agarose gel electrophoresis.

To ensure that all other possible variants are detected, patient samples negative for the FIL deletion underwent subsequent gap-PCR analyses of the 3.5-kb Thai, 114-kb Spanish, 100-kb Chinese, and 12.5-kb Vietnamese deletions using previously described PCR protocols.[17,18] These specific deletions were included based on historical data and geographic proximity to these countries.

β-Globin StripAssay Analysis

All samples were further analyzed using the reverse-hybridization technique via the β-Globin StripAssay SEA™ Kit (ViennaLab Diagnostics, Vienna, Austria) for the detection of any of the twenty-two (22) β-thalassemia variants commonly seen in the Southeast Asian population (- 31 [A>G], - 29 [A>G], - 28 [A>G], cap+1 [A>C], initiation codon [ATG>AGG], codon 8/9 [+G], codon 15 [TGG>TAG], codon 17 [A>T], codon 19 [A>G] (Malay), codon 26 [G>A] HbE, codon 27/28 [+C], IVS 1.1 [G>T], IVS 1.5 [G>C], codon 41/42 [-TTCT], codon 43 [G>T], codon 71/72 [+A], codon 89/90 [-GT], codon 90 [G>T], codon 95 [+A], IVS 2.1 [G>A], IVS 2.654 [C>T] and codon 121 [G>T]). Samples without any variants detected via StripAssay analysis were further characterized by direct sequencing of the *HBB* gene.

Sequencing of the *HBB* Gene

Direct sequencing of all three exons of the *HBB* gene was performed on all patients, excluding those with possible *HBB* gene deletions and those with compound heterozygous variants detected via StripAssay. Amplifications were done using previously described primers and PCR conditions.[18,19] Sanger sequencing was performed by Macrogen, Inc. (Seoul, South Korea) using the Applied Biosystems 3730 Genetic Analyzer. Sequencing data were compared with the reference *HBB* gene sequence (GenBank NG_000007.3) using Sequencher 5.4.6 (Gene Codes Corporation, USA).

RESULTS

Clinical Characteristics of the Study Participants

Among the 237 study participants, 126 (53.16%) were male and 111 (46.84%) were female (age range: 1 day to 64 years old; ratio: 1.15 males per 1 female). The unrelated participants were of rural and urban origins and had non-consanguineous parents. Hematological analysis (Table 1) revealed that the participants exhibited low MCH levels (MCH <27.0 pg), suggestive of β-thalassemia, as well as low Hgb levels (Hgb <136 g/L for males; Hgb <120 g/L for females), a diagnostic criterion for anemia.[20] Molecular characterization revealed that 131 patients are homozygous for a pathogenic variant, while 43 are compound heterozygous for a pathogenic variant.

Molecular Analysis

A total of 21 previously published β-globin gene variants were detected. According to their effect on gene function, ten (10) were found to be pathogenic point mutations based on their ClinVar classifications [c.79G>A (HbE) (48.52%), c.92+2T>C (1.47%), c.52A>T (0.84%), c.92+5G>C (0.63%), c.-50A>C (cap+1) (0.42%), c.79G>T (0.21%), c.59A>G (Malay) (0.21%), c.47G>A (0.21%), c.20A>T (HbS) (0.21%), and c.2T>G (initiation codon) (0.21%)]; five (5) were pathogenic deletions [*HBB* Filipino deletion (23.42%), c.203_204delTG (5.49%), c.126_129delCTTT (codon 41/42) (1.68%), c.155delC (0.63%), and c.110delC (0.21%)]; two (2) conflicting patterns of pathogenicity [c.27dup (0.42%) and c.170G>A Hb J-Bangkok (0.21%)]; and four (4) polymorphisms [c.9T>C (codon 2) (40.93%), c.315+16G>C (36.92%), c.316-185C>T (25.74%), and c.-92C>G (0.21%)]. The genotypic pattern and hematological findings of each variant are listed in Table 1.

Deletion Analysis

The FIL deletion was detected in 23.42% (111/474) of alleles, wherein 8.91% (42/474) are homozygous, and 14.60% (69/474) are heterozygous for the deletion. All were negative for the Spanish, Thai, Chinese, and Vietnamese *HBB* deletions.

β-globin StripAssay Analysis

β-globin StripAssay analysis detected 11 variants. c.79G>A (HbE) was the most common allele (48.52%), followed by the normal allele (β/β) (33.66%), then a possible deletion of the *HBB* gene (23.42%), which was confirmed to be the 45-kb FIL deletion. Other variants detected are the cd 41/42 deletion (1.68%), deletion of the wildtype of IVS 1.1 to 1.5 (1.47%), cd 8/9 insertion (0.50%), cd 17 (c.52A>T, 0.84%), IVS1.5 (c.95+5G>C, 0.63%), cap+1 (c.-50A>C, 0.42%), cd 19 (c.59A>G, 0.21%), cd 15 (0.25%), and cd 2 (ini cd) (0.21%).

Sequencing of the *HBB* Gene

Eleven (11) additional variants were detected by direct sequencing of the *HBB* gene, including three pathogenic point mutations [c.79G>T (ClinVar Accession Number: VCV000038650.105), c.20A>T (HbS) (ClinVar Accession Number: VCV000015333.149), and c.92+2T>C (ClinVar Accession Number: VCV000036334.108], three pathogenic or likely pathogenic deletions [c.203_204delTG (ClinVar Accession Number: VCV000036303.107), c.110delC (ClinVar Accession Number: RCV000586096.16), c.155delC (ClinVar Accession Number: VCV000038659.115], 1 conflicting pathogenicity point mutation [c.170G>A or Hb J-Bangkok (ClinVar Accession Number: VCV000015214.13)], and 4 benign point mutations [(c.9T>C (ClinVar Accession Number: VCV000193106.37); c.315+16G>C (ClinVar Accession Number: VCV000256345.29); and c.316-185 C>T (ClinVar Accession Number: VCV000036316.22),

Table 1. Summary of Variants Found in the HBB Gene of Filipinos with β-thalassemia

Variant NM_000518.5(HBB)	Zygosity	Allelic Frequency (n=474)	% Allelic Frequency	Clinical Significance from ClinVar	RBC (x10^{12})/L	MCV (fl)	MCH (pg)	MCHC (g/L)	Hgb (g/L)	Reference
c.79G>A (p.Glu27Lys) n = 139	homozygous	182	48.52	pathogenic	1.80-5.52	59.30-98.90	18.00-36.40	275.00-398.00	32.00-181.00	[21]
	heterozygous	48								
c.9T>C (p.His3=) n = 126	homozygous	136	40.93	benign	1.70-6.00	53.80-105.50	17.30-36.40	275.00-398.00	32.00-108.00	[22]
	heterozygous	58								
c.315+16G>C n = 111	homozygous	128	36.92	benign	1.70-7.50	53.50-105.50	17.30-36.40	281.00-398.00	43.00-108.00	[23]
	heterozygous	47								
c.316-185C>T n = 81	homozygous	82	25.74	benign	0.89-7.49	53.50-105.50	18.00-36.40	275.00-398.00	32.00-108.00	[23]
	heterozygous	40								
c.203_204delTG (p.Val68fs) n = 17	homozygous	18	5.49	pathogenic/ likely pathogenic	1.70-3.00	78.00-83.30	25.50-29.30	326.00-350.00	43.00-90.00	[24]
	heterozygous	8								
c.126_129delCTTT (p.Phe42fs) n = 6	homozygous	4	1.68	pathogenic/ likely pathogenic	0.89	73.50	23.60	321.00	483.00	[25]
	heterozygous	4								
c.92+2T>C ***(Deletion of IVS 1.1 to 1.5)*** n=6	homozygous	2	1.47	pathogenic	-	-	-	-	-	[26]
	heterozygous	5								
c.52A>T (p.Lys18Ter) n = 4	homozygous	0	0.84	pathogenic	4.74	90.10	30.50	339.00	145.00	[27]
	heterozygous	4								
c. 155delC (p.Pro52fs) n=2	homozygous	2	0.63	pathogenic	-	-	-	-	-	[28]
	heterozygous	1								
	heterozygous	0								
c.92+5G>C n = 3	homozygous	0	0.63	pathogenic	4.77	67.40	20.60	306.00	98.00	[29]
	heterozygous	3								
c.27dup (p.Ser10fs) n = 1	homozygous	2	0.42	pathogenic/ uncertain significance	-	-	-	-	-	[30]
	heterozygous	0								
c.-50A>C n = 1	homozygous	2	0.12	pathogenic	-	-	-	-	-	[31]
	heterozygous	0								
c.-92C>G n=1	homozygous	0	0.21	benign	-	-	-	-	-	[32]
	heterozygous	1								
c. 110delC n=1	homozygous	0	0.21	pathogenic/ likely pathogenic	-	-	-	-	-	[33]
	heterozygous	1								
c.59A>G (p.Asn20Ser) n = 1	homozygous	0	0.21	pathogenic	3.51	94.00	26.50	281.00	93.00	[34]
	heterozygous	1								
c.20A>T (HbS) (p.Glu7Val) n = 1	homozygous	0	0.21	pathogenic	-	-	-	-	-	[35]
	heterozygous	1								
c.170G>A (p.Gly57Asp) n = 1	homozygous	0	0.21	conflicting classifications of pathogenicity	-	-	-	-	-	[36]
	heterozygous	1								
c.2T>G (p.Met1Arg) n = 1	homozygous	0	0.21	pathogenic	6.69	53.00	16.90	316.00	113.00	[37]
	heterozygous	1								
c.47G>A (p.Trp16Ter) n = 1	homozygous	0	0.21	pathogenic	4.71	91.30	36.40	398.00	171.00	[30]
	heterozygous	1								
c.79G>T (p.Glu27Ter) n = 1	homozygous	0	0.21	pathogenic	-	-	-	-	-	[38]
	heterozygous	1								
45-kb Filipino Deletion NG_000007.3:g.66258_184734del118477 n = 90	homozygous	42	23.42	pathogenic	0.89-13.80	53.80-99.20	17.30-35.30	302.00-371.00	47.00-93.00	[16, 39]
	heterozygous	69								

c.-92C>G (ClinVar Accession Number RCV000508112.3)] (Table 1).

It is suspected that two variants detected via *HBB* gene sequencing are related to variants found via the β-globin strip assay analysis, namely, c.20A>T and cd 8/9 insertion, as well as c.92+2T>C and deletion of the IVS 1.1 to 1.5 wildtype. The pathogenic mutation c.79G>T was found to be heterozygous, coinciding with c.79G>A (HbE).

DISCUSSION

The earliest studies on the molecular basis of β-thalassemia in Filipinos were done overseas among Filipino migrants in Canada and Taiwan. A 45-kb Filipino gross genomic deletion that resulted in the removal of the entire *HBB* gene and β-thalassemia major was previously identified in two Filipinos living in Canada.[16,24] Another study in Taiwan reported three variants, namely, (1) the 45-kb Filipino deletion, (2) c.203_204delTG [codon 67 (-TG)], and (3) c.79G>A (HbE), which accounted for 92% of thalassemia alleles in 2,954 Filipinos.[40] These findings show the significance of molecular studies for β-thalassemia in the Filipino cohort.

These previously reported variants were also identified in this study, such as c.79G>A (HbE, 48.52%), FIL deletion (23.42%), and c.203_204delTG (5.49%), with HbE as the most common pathogenic variant. Upon correlation with clinical data, patients with these variants exhibit lower Hgb levels with borderline and low MCH levels (Table 1). HbE manifests a mild globin chain imbalance characterized by mild anemia, minimal morphological abnormalities of red cells, and normal red cell indices. On the contrary, FIL deletion and c.203_204delTG exhibit β-thalassemia major.[39] Geographically, HbE is prevalent in Southeast Asia, specifically in northeastern Thailand, Cambodia, and Laos, which can be attributed to its high frequency in the Philippines.[41] On the other hand, a 2018 study showed the occurrence of the FIL deletion in Sabah, Malaysia, and correlated the results to their respective haplotypes.[42] Another study in the Malaysian population also reported the presence of the FIL deletion and its co-existence with HbE, which was also observed in this study. Geographically, the Philippines and Malaysia are close countries, and both are of Malay descent, which may explain the similar genotypes observed in both countries.

Among the identified variants, nine were detected by both β-globin StripAssay analysis and *HBB* gene sequencing. This includes variants in the intervening sequences, such as the rare and pathogenic c.-50A>C (cap+1), which was found in two homozygous patients. A previous study reported that the combination of cap+1 with other β^+/β^0 thalassemia affects severity and may lead to β-thalassemia intermedia or β-thalassemia major.[43] c.92+2T>C (IVS 1-2), c.92+5G>C (IVS 1.5), and c.-92 C>G were also identified and are known to interrupt intron splicing, resulting in β-thalassemia.[44,32] Similarly, two variants causing early termination (c.2T>G and c.47G>A) were also found. Both heterozygous patients identified with these variants manifest normal RBC, Hb, and red cell indices.

Of the eleven (11) additional variants detected by Sanger sequencing, five (5) were pathogenic [c.203_204delTG; c. 155delC; c.110delC; c.20A>T (HbS); c.79G>T]. One patient is heterozygous for HbS (c.20A>T) or sickle cell hemoglobin, the most common pathogenic *HBB* variant worldwide. Patients heterozygous for HbS manifest the sickle cell trait with reduced severity of symptoms, while patients homozygous for HbS are diagnosed with sickle cell disease and have the most severe phenotypes.[45] The presence of pathogenic deletions such as c.203_204delTG and c.155delC was also detected via sequencing, including in patients with the normal genotype (β/β), based on StripAssay analysis. The c.155delC variant results in early termination of codon 60, causing β^0 thalassemia.[30] The compound heterozygosity of this variant with β^0 thalassemia and severe β-thalassemia variants, such as c.92+5G>C, causes severe clinical manifestations that eventually lead to β-thalassemia major.[46] Interestingly, a nonsense pathogenic variant, c.79G>T, was also identified and coexists with HbE (c.79G>A), resulting in pre-termination of β-globin synthesis.[40,47] The identification of these pathogenic variants further demonstrates the importance of HBB gene sequencing and highlights the limitations of the beta-globin StripAssay kit.

Among the benign variants, c.9T>C was the most common, as observed among 40.93% (194/474) of alleles, including those with normal (β/β) genotype. This variant was first reported and is predominant in Odisha State, India, and similarly, was also reported to be of high frequency among their normal population.[23] Borderline MCH and Hgb levels of patients with this variant were observed. Other prevalent SNPs identified are intronic (c.-92C>G, c.315+16G>C, and c.316-185C>T).

In this study, it is important to note that patients with a normal (β/β) genotype were observed to manifest β-thalassemia phenotypes. This may be due to a) undetected intronic variants in the *HBB* gene; b) genetic modifiers that ameliorate the β-thalassemia phenotype; or c) coinheritance with alpha-thalassemia, which are outside the limitations of this study.[12] Overall, a wide range of β-thalassemia variants were identified among 237 participants in this study, including the 45-kb FIL deletion. Although no novel variants were identified, this study has shown the importance of molecular characterization of *HBB* genes among patients with β-thalassemia, and also contributes to the identification of cost-effective methods for genotyping—sequencing and deletion analysis can already provide the necessary information for beta-globin genotyping since the variants included in the StripAssay kit can be detected via Sanger sequencing and deletion analysis can detect the presence of the 45-kb FIL deletion.

CONCLUSION

This is the first to report the unique molecular heterogeneity of β-thalassemia among affected Filipinos. The high number of variants presented here emphasizes the importance of the development and execution of national prevention programs that provide baseline molecular data for precise diagnosis, carrier screening, genetic counseling, and disease management for these patients. Detection of the FIL deletion in the majority of β-thalassemia patients shows its significance in its inclusion in the Expanded Newborn Screening Program. Identified genotypes can also serve as a guide for transfusion frequency, iron chelation policies, and timing of patient follow-up visits. For instance, patients with beta-thalassemia major (β^0/β^0) have higher rates of iron-related complications, require frequent blood tests, and/or may be advised to undergo blood transfusion and iron chelation. On the other hand, milder beta-thalassemia genotypes, such as (β^+/β^+), may only be required to undergo less frequent blood transfusions and follow-up.[48] However, a limitation of this study is the lack of information regarding the clinical presentation of participants and, subsequently, the determination of clinical severity in correlation with genotypes, given the wide age range of study participants.

Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

All authors declared no conflicts of interest.

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