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CLINICAL REPORT

The use of segregation analysis in interpretation of sequence variants in SMAD3: A case report

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Abstract

Background: While representing a significant improvement, the introduction of next-generation sequencing in genetic diagnosis also prompted new challenges. Despite widely recognized consensus guidelines for the interpretation of sequence variants, many variants remain unclassified or are discordantly interpreted. In heritable thoracic aortic aneurysms with dissection (HTAAD), most cases are caused by a heterozygous, private missense mutation, possibly contributing to the relatively common reports of variants with uncertain significance in this group. Segregation analysis necessitates advanced likelihood-based methods typically inaccessible to non-experts and is hampered by reduced penetrance, possible phenocopies, and non-availability of DNA from deceased relatives.

Methods: In this report, challenges in variant interpretation and the use of segregation analyses were illustrated in two families with a suspected HTAAD disorder. The R package segregatr, a novel implementation of full-likelihood Bayes factor (FLB), was performed to explore the cosegregation of the variants in these families.

Conclusion: Using the R package segregatr, cosegregation in the reported families concluded with strong and supporting evidence for pathogenicity. Surveillance of families in a multidisciplinary team enabling systematic phenotype description for standardized segregation analysis with a robust calculation method may be imperative for reliable variant interpretation in HTAAD.

KEYWORDS

heritable thoracic aneurysms and dissections (HTAAD), Loeys–Dietz syndrome, next-generation sequencing, segregation analysis, *SMAD3*, variant interpretation

1 | INTRODUCTION

Heritable thoracic aortic aneurysms and dissections (HTAAD) refer to a group of genetic connective tissue disorders characterized by a predisposition for severe arterial pathology. While HTAAD may be difficult to distinguish from their multifactorial phenocopies in the general population, identification of individuals at risk is crucial for surveillance and early intervention, enabling the prevention of severe vascular events and premature

death (Brownstein et al., 2018). HTAAD typically comprises autosomal dominantly inherited disorders that are often caused by private missense variants. This may contribute to the relatively frequent reports of variants with uncertain clinical significance (VUS) (Pope et al., 2019). The pathogenesis is typically linked to dysfunction of the extracellular matrix, medial smooth muscle cells, or transforming growth factor beta (TGFβ) signaling, but the effects of individual gene variants are not completely understood due to the lack of previously reported cases or ambiguous experimental evidence (Ostberg et al., 2020). Interpretation of variants may be challenging even in the presence of multiple familial cases because segregation analysis is hampered by age-dependent and reduced penetrance, abundant phenocopies in the general population, as well as non-availability of DNA from deceased relatives.

Loeys–Dietz syndrome (LDS) represents a syndromic form of HTAAD, typically affecting the vascular and skeletal systems. Largely as a result of next-generation sequencing (NGS), six types of LDS have been described, all being caused by variants in genes involved in the TGFβ pathway. LDS type 3 (LDS3) is caused by pathogenic variants in *SMAD3* (Regalado et al., 2011; Van de Laer et al., 2014), and at least 120 different pathogenic variants have been reported in this gene (Human Gene Mutation Database (HGMD) Professional 2020.4). LDS3 is autosomal dominantly inherited and has an estimated prevalence of 1:100000, frequently presenting with aneurysm disease with or without osteoarthritis and skeletal manifestations (Van de Laer et al., 2014). Aneurysms and osteoarthritis of other causes represent frequent phenocopies in the general population.

In 2015, a work group consisting of representatives from the American College of Medical Genetics and Genomics (ACMG), the Association for Molecular Pathology (AMP), and the College of American Pathologists (CAP) published consensus guidelines for classification of sequence variants in Mendelian and mitochondrial disease. According to the guidelines, classification is based on the combination of different types of evidence including populational, computational, functional, and segregation data (Richards et al., 2015). However, it has been documented that these classification criteria are discordantly interpreted by different laboratories even when applying the guidelines. (Amendola et al., 2016, 2020).

Regarding the use of segregation analysis, Richards et al. (2015) admitted that statistical evaluation of co-segregation may be difficult in the clinical laboratory setting. The most comprehensive method involves computing a full-likelihood Bayes factor (FLB) (Thompson et al., 2003), requiring the use of advanced linkage software. In response to this, a simpler alternative based on meiosis counting was suggested (Jarvik & Browning, 2016) that gives a good approximation of FLB in many cases. However, in complex cases, for example, with reduced penetrance or sparse DNA availability, the counting method has been shown to be inadequate (Rañola et al., 2018). In HTAAD, premature deaths may limit the number of genotyped individuals. Also, some families do not have affected members spanning through several generations due to de novo pathogenic variants.

Further, according to the ACMG/AMP/CAP guidelines, segregation may count as either supporting, moderate, or strong evidence depending on the extent of segregation. Unfortunately, the term "extent of segregation," or criteria for extensive segregation, was not defined.

2 | METHODS

In the current report, the ACMG/AMP/CAP guidelines (Ref Richards) were used for the interpretation of two sequence variants of uncertain significance in *SMAD3* (NM_005902.3, OMIM# 603109). To explore co-segregation of these variants and LDS3 phenotypic traits, a novel implementation of the FLB method (Vigeland, 2021) was used. Single-family thresholds adapted from Jarvik and Browning (2016) were applied to convert FLB scores to ACMG/AMP/CAP evidence classes. The R package segregatr, part of the ped suite packages for pedigree analysis in R (Vigeland, 2021) was applied to compute the FLB for each family. The implementation is based on the Elston-Stewart algorithm for pedigree likelihoods (Elston & Stewart, 1971). In order to convert FLB scores to ACMG evidence classes we adapted the single-family thresholds given by Jarvik and Browning (2016). This implies that the FLB thresholds for supportive, moderate, and strong evidence are 8, 16, and 32, respectively.

The segregatr R package is freely available from The Comprehensive R Archive Network (https://CRAN.R-project.org/package=segregatr). Source code for the FLB calculations and plots is given in the Supporting Material.

3 | CASE DESCRIPTION

The index of family A had been suffering from osteoarthritis since his teens. At the age of 46, he was admitted to the local hospital for dyspnea and fatigue. He was diagnosed with chronic obstructive pulmonary disease, and dilatation of the ascending aorta was revealed. It was also noted that he had a marfanoid habitus, hypermobile joints, and an inguinal hernia. The patient was the youngest of several siblings, of whom two were operated for aortic aneurysms at the time. His father and paternal grandfather were tall with long extremities including the

fingers. Sanger sequencing and MLPA analysis of *FBN1* did not uncover any likely pathogenic variant in Index A. He also did not fulfill the diagnostic criteria for Marfan syndrome (Loeys et al., 2010). At the age of 48, an infrarenal abdominal aneurysm was diagnosed by CT.

The patient was referred to a consultation with a clinical geneticist. Given the patient's clinical history and family history, an NGS was performed using an Illumina platform for exome sequencing with bioinformatic filtering for 34 genes (first edition) that were all associated with hereditary connective tissue disorders (HCTD). Three novel VUS were reported; one in *FBN2* (OMIM# 612570), c.4102G>A (p.Val1368Met) (PP3-supporting), where pathogenic variants lead to congenital contractural arachnodactyly and early onset macular degeneration, another in *PLOD1* (OMIM# 153454), c.1927G>A (p.Val643Ile), where pathogenic variants lead to the autosomal recessive, kyphoscoliotic type of Ehlers–Danlos syndrome, and a third, c.269G>A (PM2-supporting and PP3-supporting), in *SMAD3*, which is the gene for LDS3. Out of the three mentioned disorders, only LDS3 was found to fit with the patient's phenotype. The reported amino acid change in *SMAD3*, (p.Arg90His), was localized in a functional domain that is well conserved in different species as well as in other proteins in the SMAD family. The specific variant had not been recorded in databases with healthy subjects (Exome Aggregation Consortium (ExAc) which was the database that was used at the time of the variant assessment). The laboratory-recommended segregation analysis. Altogether, 13 relatives were assessed and tested for the variant, the results being presented in Figure 1. Upon re-interpretation of the variant in the diagnostic laboratory, the results of the segregation analysis were considered as supporting evidence for pathogenicity, but the variant remained unclassified.

Years after the first investigations, a new scientific article presented several *SMAD3* variants as likely pathogenic or pathogenic, including the one found in family A (Schepers et al., 2018). In the published case, segregation was interpreted as moderate rather than supporting evidence of pathogenicity, which resulted in classification of the variant as likely pathogenic. In the same year, this variant was reported in another article (Hicks et al., 2018), stating that the family history supported pathogenicity. Considering the published reports of unrelated cases, the variant in family A was eventually classified as likely pathogenic (PP3-supporting, PM2-supporting, PP1-moderate, PM1-moderate).

In the index of family B, during investigation of respiratory symptoms dilatation of the aortic root was revealed at the age of 28. Later at the age of 36, index B was diagnosed with left ventricular dilatation. This prompted genetic investigation. Thirteen genes associated with arrhythmogenic right ventriculocardiomyopathy and dilated cardiomyopathy were Sanger sequenced with negative results. Index B's early onsetting symptoms and

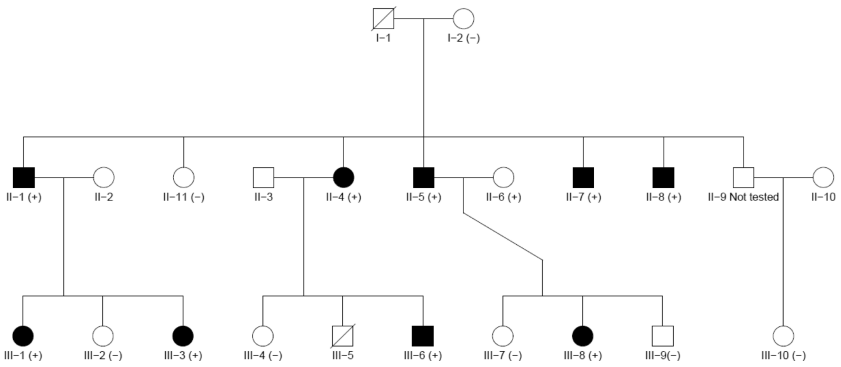

FIGURE 1 Family A. *SMAD3*: c.269G > A. II-1: Aortic root dilatation measured at 4,1 cm (34), the aortic root dilatation had progressed to 5,1 cm causing an aortic valve insufficiency (50), valve and conduit replacement (53). II-4: Osteoarthritis, cardiac examination uncovered an ectasia in the upper part of the descending aorta measuring up to 3,3 cm (63), no progression in the ectasia at last checkup (66). II-5: Pectus excavatum, supraventricular tachycardia since childhood, pulmonary embolism (36), asthma, dilated aortic root causing aortic valve insufficiency and mitralprolapse (43), mitral valvuloplasty (58), the aortic root dilatation progressed to 5,1 cm (61) and was operated, kidney cancer (48), arterial tortuosity, COPD, anterior communicating artery aneurysm measured 5 mm (60), which was coiled due to progression in size (64). II-7: Dilation of Valsalva sinus measured 4,4 cm (54), conduit replacement (55), aneurysm of the iliac artery communis 24 × 40 mm (54), tortuosity of the carotid arteries. II-8: Inguinal hernia, osteoarthritis (16), aortic root dilatation measured 46 mm, saccular aneurysm in kidney arteries 26 mm and saccular aneurysm in lumbal aorta 26 × 29 mm (49), operated at 52 and 55. COPD. III-1: Tortuosity of the carotid arteries, aortic root measured to 36 mm at last checkup (49). III-3: Tortuosity of the carotid arteries and intracranial arterial fenestration (35), no progression at last checkup (40). III-6: Ectasia of iliac artery communis measuring 18 mm diameter, ectasia of the mammary artery measuring 8 mm in diameter, a dilated aortic root measuring 45 mm (40), operated with conduit replacement and valvuloplasty (41). III-8: Migraine, varicose veins, dilated aortic root measuring 40 mm (33), conduit replacement because of progression of root dilatation to 44 mm (35), complicated by chronic pericarditis.

the family history indicated an HTAAD (Figure 2), and NGS was performed with bioinformatics filtering of the same 34 genes as in the index of family A. A sequence variant of uncertain significance, c.1243G>C (p.Gly415Arg) (PM2- supporting, PP3-supporting), in *SMAD3* was reported. It was not annotated in the control database, ExAc, and it altered a well-preserved amino acid in a recognized protein family. The amino acid is conserved between species and is located in a domain with several important functions. However, the high degree of conservation was noted only as supportive evidence for pathogenicity, and PM1 was not used as the affected domain covers 45% of the protein and therefore considered unspecific. As sufficient evidence was lacking it was concluded that the variant was of unknown significance for disease. Segregation analysis in family B was approached through genetic counseling and testing of adult relatives with vascular manifestations (Figure 2). The results suggested co-segregation of the variant and disease, but this was not considered sufficient to be weighed higher than supportive evidence for pathogenicity. Later, the same variant was reported as pathogenic in an independent family (Schepers et al., 2018). Because of stringent interpretation of the ACMG criteria, a new re-interpretation of the variant was requested but concluded that the variant remained a VUS (PM2-supporting, PP3-supporting, and PS4-supporting).

In families A and B, all first-degree relatives of patients with suspected HTAAD were offered genetic counseling and surveillance in a multidisciplinary clinic for HTAAD that has an approved quality register based on informed consent. Here, the phenotype descriptions were refined by consultants with relevant expertise, and segregation analyses were pursued in a longitudinal approach.

4 | CONCLUSION

The suggestion that specific disease group experts should continue to develop adapted evidence regarding the classification of variants in specific genes because the applicability and weight assigned to certain criteria may vary by gene and disease (Richards et al., 2015) has already been addressed for certain groups (Moghadasi et al., 2016; Rosenthal et al., 2017). The cases in the present report illustrate the relevance of such an action in the group HTAAD that frequently presents with sequence variants of uncertain significance (Pope et al., 2019). In both families presented, additional evidence was required for classification with respect to the likelihood for pathogenicity. Functional studies were not available, and segregation analysis was recommended in both cases.

The reason for discordant classification of the variant in family A at two laboratories seemed to be that the criterion, "co-segregation with disease in multiple affected family members in a gene definitely known to cause the disease" was acknowledged as supporting evidence in one laboratory and as moderate evidence in the other. Similar challenges in interpreting the family history could explain the discordant variant interpretation in family B.

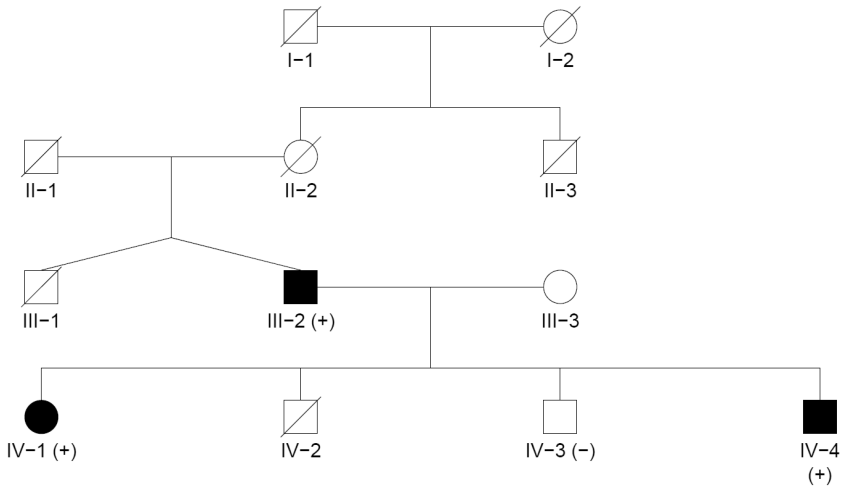

FIGURE 2 Family B. *SMAD3*: c.1243G > C. II-1: Died of presumably stroke (68). II-2: Died of cancer (67). II-3: Died suddenly, unexplained cause (54). III-1: Died in an accident, fell from height (37). III-2: Osteoarthritis, operated for mitral valve insufficiency (44) and aortic valve insufficiency (51), conduit replacement due to 60 mm aortic dilatation (65). IV-1 (INDEX): Migraine, celiac disease, osteoarthritis, pectus carinatum, bifid uvula, dilated aortic root 42 mm (32), dilated left ventricle (36), conduit replacement due to progression of root dilatation with valvuloplasty (39). IV-2: Died of a ruptured aortic aneurysm (26). IV-4: Scoliosis, hypermobile joints, inguinal hernia operation (6 and 7), slightly dilated aortic root measuring 38 mm (19), conduit replacement due to progression of root dilatation to 41 mm (27), slight/medium aortic insufficiency (32).

While we cannot claim that any of these interpretations were incorrect, the example illustrates that discordant interpretations between laboratories may stem from different application of the codes for evidence (Amendola et al., 2020). In family A, acknowledgment of reports of unrelated families eventually resulted in concordance. In family B, the report was not considered sufficient and the classification remained discordant.

According to the ACMG/AMP/CAP guidelines, clinical laboratories are encouraged to work with experts in statistics or population genetics to ensure proper modeling in segregation analysis and avoid incorrect conclusions of the relevance of the variant to the disease (Richards et al., 2015). Correct calculation alone cannot solve the issues with segregation analysis in HTAAD. Quantitative methods such as counting meiosis rely on affected carriers as the sole units of information and other phenotypic and genotypic properties are not taken into account (Rañola et al., 2018). As the present cases illustrate, reduced or age-dependent penetrance and possible phenocopies make it hard to assess which relatives may be affected by the disorder and which may not (Figures 1 and 2).

Taking these points into account, we pursued a more robust calculation method for segregation using the FLB method that concluded with strong and supporting evidence for pathogenicity, respectively.

In family A, using a dominant disease model with 90% penetrance, 1% phenocopy rate, and disease allele frequency 1e-5 resulted in an FLB of 522, clearly indicating *strong* evidence for pathogenicity. Recognizing the uncertainty in the chosen parameters, we also repeated the computation several times with more conservative parameters, always resulting in a score well above the threshold FLB = 32. In particular, a contour plot of FLB as a function of the penetrance and the phenocopy rate shows strong evidence for all realistic parameter values (Supporting Figure S1a) indicating that this is a robust conclusion. In family B, using the same parameters as for family A, a segregation score of FLB = 13 was obtained, amounting to *supportive* evidence for pathogenicity. Again, varying the parameters consistently gave the same result (Supporting Figure S1b). In conclusion, the evidence provided by segregation analysis implied an overall variant classification as *likely pathogenic* for the variants in family A. In family B, the original conclusion regarding segregation evidence was upheld by the reanalysis.

Erroneous classification of variants could be fatal in HTAAD. While awaiting improved knowledge on molecular pathogenesis and availability of functional studies, quantification of the evidence criterion, and segregation should be obtained with standardized analysis and robust calculation. In some cases, the acknowledgment of unrelated patients with the same variant in publications or international databases result in the classification of the variant. Although sharing of interpreted variants may aid concordant classification, the evidence for the interpretation is frequently not presented in detail. When stating that a specific variant is segregating with the disorder in a family, it would be helpful if more detailed information on the basis for segregation is shared.

We conclude that the implementation of the FLB method with single-family thresholds was helpful in performing robust segregation analyses. Further, we expect that systematic clinical investigation and longitudinal surveillance of families with a VUS in a relevant gene in a multidisciplinary clinic using standardized diagnostic methods and an approved patient register may improve the clinical data for segregation analysis that may aid classification of genetic sequence variants in HTAAD.

AUTHOR CONTRIBUTIONS

All authors contributed substantially to conception and design of this article, critically revision of important intellectual content, and final approval of the version to be published. Aleksandra Ratajska drafted the article. Magnus Vigeland performed segregation analysis. Aleksandra Ratajska, Katrine Verena Wirgenes, Kirsten Krohg-Sørensen and Benedicte Paus contributed to acquisition of clincal data.

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None.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest related to the content in this report.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the report.

ETHICS STATEMENT

No ethical issues were identified for this article.

PATIENT CONSENT

All patients presented in this manuscript have provided informed, written consent to publication of their genotypes and phenotypes.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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