The Phenotypic-to-Genotypic Association of Novel Single-Nucleotide Polymorphisms in the Collagen Matrix-Encoding Gene ZNF469 in Arterial Aneurysmal Diseases

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Abstract

Research Question: In patients with a strong personal and or family history of aortic and or aneurysmal events, in the absence of known syndromic mutations, is there be a genetic basis for disease?

Background, Significance, and Rationale: Aneurysms and dissections are devastating vascular pathologies which are increasingly recognized to have a genetic basis. In this report we share novel mutations in ZNF469 in eight patients in our practice with vasculopathy, such as aneurysm, ectasia, and/or dissections. This gene is responsible for the production of a collagen-related zinc-finger protein involved in multiple aspects of the development and regulation of major extracellular matrix components.

Methods: Patients with significant personal or familial history of aneurysmal or dissection diseases were genotyped to assess mutation status of genes associated with vasculature, extracellular matrix, and aneurysmal/dissection disease.

Results: All patients were positive for ZNF469 mutations, with 7/8 being positive for variants of unknown significance, and 1/8 positive for a pathologic mutation indicated in development of brittle cornea syndrome. Of the eight patients tested, 5/8 (62.5%) have ectasia/aneurysmal disease, 3/8 (37.5%) have experienced vascular dissection, and 4/8 (50.0%) have a family history of 1 or more first-degree relative with aneurysmal or dissection disease.

Conclusions: We recently reported the first known case associating this genotype to phenotype, and this work significantly extends this discovery by associating polymorphisms in this gene with vasculopathies in a significant cohort of unrelated patients. Furthermore, the co-segregation of these mutations exclusively within exon 1 and exon 2 of this gene suggests the importance of these exons in the genetic architecture of ZNF469 as it relates to human disease.

Research Question

In patients with a strong personal and or family history of aortic and or aneurysmal events, in the absence of known syndromic mutations, is there be a genetic basis for disease? The goal of this study was to determine if such patients possessed mutations in vascular or extracellular matrix-related proteins that may be causative for their pathologies. It was our hypothesis that a genetic mutation would be present, likely driving their disease states and that of their family members.

Introduction, Significance, and Rationale

The extracellular matrix (ECM) is a complex network of interlinked macromolecules, organized depending on tissue type, to carry out many dynamic and varying functions, and any dysfunction may produce catastrophic consequences. Mammalian ECM are composed of around 300 proteins, with elastin, glycoproteins, collagen, and proteoglycans being major constituents, allowing the ECM to display wide array of biochemical and physical properties [1]. ECM structure can vary such that it exists in the blood as plasma with a tensile strength of ~50Pa all the way up to 2-4GPa in bone [2]. This myriad of components not only provide structural support, but allow the ECM to aid in the carrying out of critical cellular functions, such as migration, polarity, differentiation, proliferation, and apoptosis in response to endogenous and exogenous stimuli [3]. In the vascular system of humans the tunica intima, made up of a single layer of endothelial cells and elastic lamina, plays an important role in allowing laminar flow of blood, but has little contribution to the structural and mechanical properties of the vessel, which are in large part determined by the tunica media [4]. The tunica adventitia, the outermost layer, is collagen-rich, helping to resist arterial-pressure induced vascular rupture.

The ECM has been identified in the pathogenesis of many vascular diseases whether through physiologic responses to injury, such as in hypertension-induced remodeling, or through dysfunction, such as in cystic medial necrosis-mediated aortic aneurysm [5]. Complications of ECM dysfunction resulting in aneurysmal disease, defined here as an aortic axial diameter of >4.5cm or diameter 1.5x centimeters greater than

expected, can lead to spontaneous dissection, which is often life threatening. Arterial dissections arise as an intimal tear allowing high-pressure flow into the intimal-medial aortic space, often allowing communication between the true and false lumen [6]. Studies suggest that up to 40% of dissection patients will develop a post-dissection aneurysm [7]. The general pathophysiology of aortic aneurysm involves weakening of a portion the vessel wall, namely medial degeneration with smooth muscle depletion, elastin fragmentation, and erratic proteoglycan and collagen production, often intermixed with inflammatory infiltration [8].

Mutations in 12 specific ECM genes have been identified as pathogenic and syndromic in aneurysmal disease, including classic mutations in fibrillin gene *FBN1* (resulting in Marfans), *COL3A1* (resulting in type 4 vascular Ehlers-Danlos syndrome (vEDS)), as well as less recognized genes such as those coding for various forms of collagen (*COL-1A2, -5A1-5A2*), lysyl oxidase (*LOX*), microfibril-associated glycoproteins (*MFAP5, EMILIN1*), tropoelastins (*ELN*), biglycan (*BGN*), and fibulin (*EFEMP2*) [9]. Genetic, non-syndromic predispositions to these deadly pathologies are evolving in importance as past familial studies suggest that up to ~20% of non-syndromic thoracic aortic aneurysm and dissection (TAAD) patients referred for aortic repair had an affected family member [10,11]. The idea of a single gene as a non-syndromic cause of TAAD disease is now well established with 35 individual genes currently recognized in genetic screening for familial TAAD predisposition and disease [9,11]. With greater understanding of rare and common genetic contributors of TAAD, screening for markers for genetic predisposition and determination of polygenic risk score (PRS) for aneurysmal and dissection disease will increasingly be an important aspect of cardiovascular work-up, especially in relatives of effected individuals.

In this study we report for the first time a cohort of 8 *non-syndromic* cases of aortic aneurysms and/or arterial dissections in unrelated patients with a family history of at least one other affected member who have novel mutations in ZNF469.

ZNF469 – A Collagen Matrix Protein

ZNF469 is a poorly described ECM gene with a collagen-related protein product called Zinc Finger Protein 469, and mutation of which is currently only indicated in the pathogenesis of brittle cornea syndrome, an autosomal recessive connective tissue disease of the eye resulting in a thinned cornea, keratoconus, skin hyperlaxity, and joint hypermobility, but is notably absent in the literature of vasculopathy [12-15]. Recently, our group identified ZNF469 for the first time reported as a novel gene associated with arterial aneurysmal disease [16]. Though originally it was thought to be a single- or double-exon gene, it is currently recognized as a 5-exon gene with hundreds of potential enhancer and promotor sequences, though only the first two exons are recognized as coding-exons [17,18]. ZNF469 belongs to the zinc-finger protein-producing gene family that is one of the most common motifs in eukaryotes. Multiple ZNF proteins are known to be present in vascular development and pathology, and ZNF469 likely plays a role, as well [19]. In a murine knock-out model, Stanton et al. demonstrated that an induced loss of function mutation in ZNF469 lead to significant decrease in biomechanical strength of the cornea, likely via ECM disruption [20]. The ECM of vasculature plays a critical role in both structure and function, and to date, induced mutations in ZNF469 have been shown to affect the production of several important ECM components, including Collagens I and III, Thrombospondin I (TMBSP1), and Clusterin, and shares 30% homology with Collagen I [15,17].

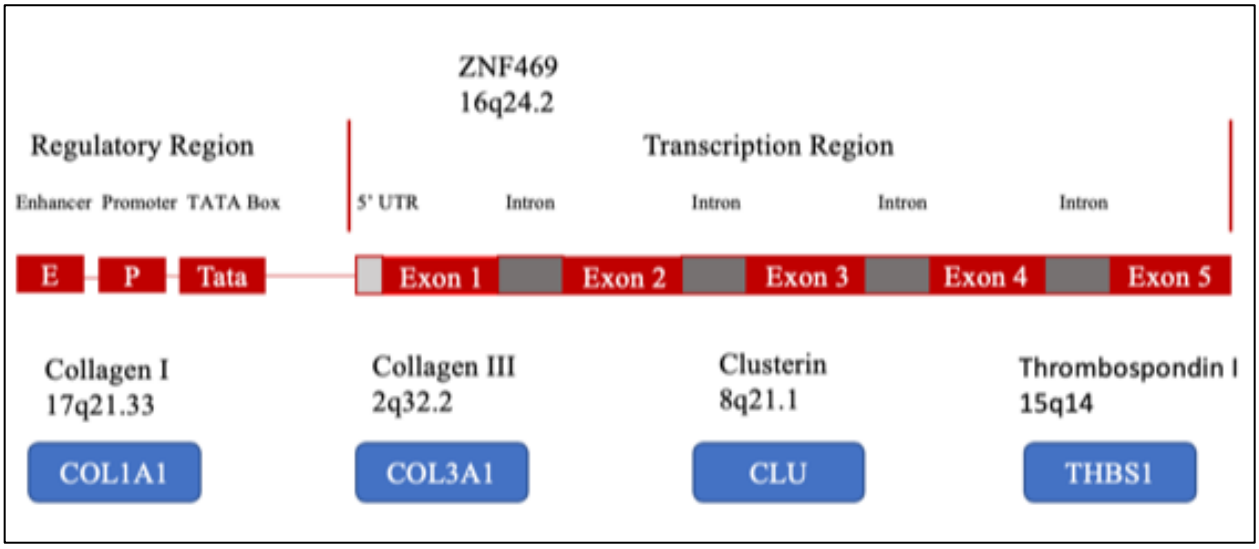

Figure 1. Position and proposed gene structure of ZNF469, and potential downstream targets.

Collagen type I is a heterotrimer consisting of two α1chains and a single α2 chain, while type III, a homotrimer, consists of three α1 chains. Collagen is the most abundant protein in animals, and is a major constituent of all type of vasculature, as types I and II account for roughly 90% of all arterial collagens [21]. Unsurprisingly, both subtypes have been implicated in multiple disorders with vascular manifestations, including vEDS and Osteogenesis imperfecta [22,23]. ZNF469 thought to potentially act as a regulator of collagen fibers transcription and or synthesis, and current research indicates that mutations in ZNF469 can cause collagen dysregulation in multiple ways, including downregulation of COL4A1 (collagen I), COL11A1 (collagen XI), collagen receptor dysfunction, and clinically in the form of thinning of collagen fibrils [17,24,25].

TMBSP1 is a glycoprotein known to interact with many ECM components and is considered a potent inhibitor of angiogenesis [26,27]. The role of TMBSP1 in blocking angiogenesis is multifactorial but involves vascular endothelial growth factor (VEGF) antagonization and upregulation of pro-apoptotic pathways in endothelial cells. Interestingly, murine models have demonstrated that maladaptive changes in TMBSP1 signaling played a central role in the development of TAAs via disruption of cytoskeleton remodeling and disruption of elastin, as demonstrated by Yamashiro et al [28]. Given the interactions of ZNF469 and TMBSP1, this may play a role in the development of TAAD in patients with ZNF469 mutation.

Clusterin, or Apolipoprotein J, is a glycoprotein with diverse function that has been indicated in the pathogenesis of many diseases ranging from cancer to Alzheimer's disease and cerebral amyloid angiopathy. It has been shown to play an important role in vascular smooth muscle cell (VSMC) differentiation, phenotype modulation, and formation of VSMC nodule formation [29]. Shirasawa et al demonstrated via mouse model that clusterin-deficient mice significantly reduced neointimal hyperplasia post-vascular insult via inhibition of VSMC proliferation and VSMC cell-cycle arrest [30]. The role of ZNF469 mutation and development of TAAD disease requires further investigation.

Methods

After receiving human subject study approval from TCU (IRB#2022-106), we consented, interviewed, and performed a detailed EMR chart review of each subject who possessed a mutation in ZNF469 at Consultants in Cardiovascular Medicine – Fort Worth PLLC (CCMS-FW). All patients had undergone genetic testing in CCMS-FW as a clinical recommendation to guide clinical decision making in their care. Genetic counseling was provided on a 1:1 basis with each patient in our practice.

Subjects underwent molecular screening of a 35 TAAD-gene panel, associated with dissection and aneurysmal disease and related disorders through genomic deoxyribonucleic acid-isolated samples obtained via saliva. Bait-capture methods were utilized for enrichment of coding exon sequences of interest using biotinylated oligonucleotide probes and subsequent polymerase chain reaction and sequencing, utilizing NCBI reference sequences [31].

Results

Mutations in Exon 1

SNP p.T910I: A 37-year-old Caucasian-American male with a past medical history of hypertension presented for management of treatment resistant hypertension. The patient was started on metoprolol and underwent a trans-thoracic echocardiogram (TTE) showing normal left atrial and ventricular dimensions with a bicuspid aortic valve (AV) (11 o'clock and 4 o'clock orientation) with no aortic insufficiency (AI) or stenosis (AS). The patient's maternal uncle developed an ascending aortic aneurysm at the age of 63. Given this patient's bicuspid valve and a family history significant for aortic aneurysm, we proceeded first with a CT-angiogram (CTA) that revealed an ascending aorta measuring 3.4 cm in diameter. We then offered him genetic testing, which revealed a heterozygous mutation VUS of p.T910I in the ZNF469 gene with Grantham Score (GS) of 89 (similar amnio acid substitution) in a position that contains Isoleucine in

other vertebrates as a reference [32]. The variant p.t9101I (also known as c.2729C>T) is considered tolerated by *in silico* analysis [32].

SNP p.G1933V: A 76-year-old Caucasian-American female self-referred to our practice for an opinion on an abnormal echocardiogram performed out of state. After review of records, a TEE was performed for definitive valvular characterization revealing an aortic aneurysm measuring 4.3 to 4.4 cm with a subsequent aortic CTA demonstrating a maximal measurement of 4.3 cm. To guide longitudinal clinical management in our aortic practice, we offered her genetic testing which revealed a heterozygous mutation VUS of p.G1933V in ZNF469 with a GS of 109 (dissimilar amnio acid substitution) in a position that is poorly conserved in vertebrates [33]. The p.G19333V variant, also known as c.5798G>T, is considered tolerated by *in silico* analysis.

SNP p.V271D: A 45-year-old Caucasian-American female presented for cardiovascular consultation with us after hospitalization for bilateral vertebral artery dissections in 2020. The patient had no prior cardiovascular history and was in good health leading up to her vascular event; she experienced a rapid-onset occipital headache that was unrelieved by non-steroidal anti-inflammatory agents. The headache gradually subsided, but four days later she experienced unilateral weakness of her right lower extremity, upper extremity, along with dysarthria and mild ataxia. She was brought to the emergency department and underwent non-contrast CT that revealed no hemorrhagic abnormalities. The symptoms mainly subsided over the course of an hour and subsequent MRA six hours later displayed bilateral vertebral artery dissection. The patient underwent urgent inpatient neurovascular consultation, with the decision made to medically manage her case with aspirin and clopidogrel. Minimal residual paresthesia of her right upper extremity was her lone lingering effect at the time of our consultation. A six-month follow-up MRA revealed chronic occlusions of the bilateral vertebral arteries with compensated collateral flow; we have referred her for another neuro-interventional opinion. Our patient revealed that her mother expired from a ruptured cerebral artery aneurysm at the age of 55, and her son has joint hypermobility and is undergoing

a genetic evaluation. Given the patient and family history of aneurysmal disease and connective tissue abnormalities, we offered her a genetic investigation. This revealed a p.V271D heterozygous mutation VUS in ZNF469, with a GS of 152 (highly dissimilar amnio acid substitution) in a position that is poorly conserved in vertebrates [34]. The p.V271D variant (also known as c.812T>A) is predicted to be tolerated by *in silico* analysis [34].

C.2193delG: A 25-year-old Caucasian-American male presented in 2021 for cardiovascular evaluation of connective tissue disease risk. On physical examination had a marfanoid appearance including height of 6'5", weight of 160 lbs., pectus excavatum, and hypermobility/laxity of multiple joints. His electrocardiogram demonstrated sinus rhythm with no pro-arrhythmic features. His TTE demonstrated an ejection fraction greater than 70%, and normal appearing ascending aortic diameter and caliber, with a bicuspid AV angulated at 3 o'clock and 9 o'clock without AI or AS. Given the patient's presentation, we offered genetic testing, which revealed a C.2193delG mutation in the ZNF469 gene, a pathogenic mutation in exon 1that is thought to cause a frameshift, with a predicted premature stop codon (p.T732Pfs*68) [35]. CTA of his chest demonstrated fusiform ectasia of the proximal descending thoracic aorta measuring 3.3 cm. His mutation was considered pathogenic for Brittle Cornea Syndrome, for which he is undergoing corneal specialty ophthalmology consultation [35].

Mutations in Exon 2

p.R1875C: A 61-year-old Caucasian-American female with no personal cardiac history or risk factors experienced a spontaneous coronary artery dissection (SCAD) of the left anterior descending coronary artery in 2014. The patient was started on a beta-blocker and discharged after stabilization. Given the patient's SCAD and a similar history in her sister, we offered her genetic testing. This revealed a p.R1875C variant in ZNF469, which is considered a mutation VUS with a minor allele frequency (MAF) of 0.018%

and a Grantham Score of 180 (highly dissimilar amnio acid substitution) [36]. The p.R1875C variant (also known as c.5623C>T) is predicted to be tolerated by *in silico* analysis [36].

p.Q3094R, p.G2871S, and p. S2637T: A 72-year-old African-American female underwent a bioprosthetic AV replacement in 2015, and at that time her aorta was normal in dimension and appearance. In 2019, she presented to her primary care physician (PCP) with abdominal pain, and subsequent CTA of her aorta with bilateral runoff demonstrated chronic, bilateral distal aortoiliac dissections that were repaired with endovascular stenting at our institution. She subsequently underwent a TEE to re-evaluate her AVR in 2021 that demonstrated enlargement of the ascending aorta to 4.5cm. We probed her family history further and learned of a significant history of aortic diseases and dissection in her extended family, allowing us to generate a family pedigree (Figure 2). Based on her history and this pedigree, we offered her genetic testing. This revealed three heterozygous mutation VUSs in exon 2 of the ZNF469 gene: p.Q3094R, p.G2871S, and p. S2637T. The variants in the ZNF469 p.G2871S and p.S2637T produce GSs of 56.00 (similar amnio acid substitution) and 58.00 (similar amnio acid substitution), respectively, while the p.Q3094R substitution yields a GS of 43.00 (highly similar amnio acid substitution), and all mutation take place in positions that are poorly conserved in vertebrates [31]. The p.QS094R variant (also known as c.9281A), p.G2871S variant (also known as c.8611G>A), and the p.S2637T variant (also known as c.7909T>A) were all predicted to be tolerated by *in silico* analysis [31]. We previously reported this compound heterozygote case as the first known case in this genotype-phenotype association of ZNF469 to vascular aneurysmal disease [16].

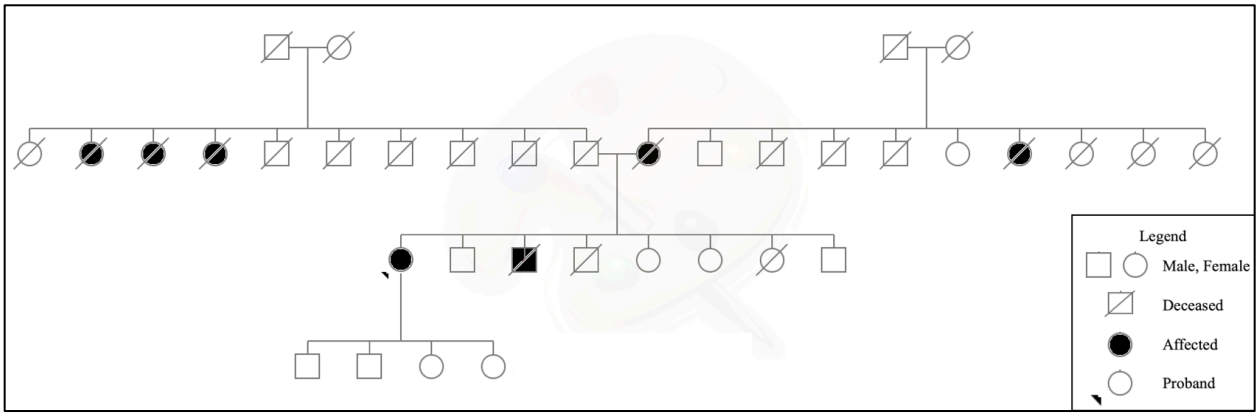

Figure 2. Family Pedigree of Aneurysmal and Dissection Disease. Affected members indicate a phenotypic endpoint of aneurysmal or dissection disease. Patient-reported and detailed family history of familial vascular dissection disorders. Analysis of this pedigree, in conjunction with the heterozygosity of the mutations, suggests a Mendelian pattern of AD inheritance with incomplete penetrance, if we assume that the first generation noted likely had disease without detection in the late 1800's. A more in-depth analysis of the genetics in relation to molecular impact is warranted to further elucidate the nature of the penetrance and molecular impact of such mutations correlating with varying phenotypes.

p.S2646F: A 56-year-old Asian-American female presented with a past medical history of hypertension, dyslipidemia, and T2DM and a familial history of aneurysmal disease presented for management of treatment resistant hypertension. Though she had no known arterial aneurysmal disease, the patient's mother developed an ascending aortic aneurysm discovered at the age of 78, a maternal aunt expired at the age of 40 secondary to a vertebral artery aneurysm rupture, and several other extended family members had aneurysms. Given this history, we proceeded with genetic investigation. This revealed a heterozygous mutation VUS p.S2646F (poorly conserved position in vertebrates) in ZNF469 gene with a GS of 155 (highly dissimilar amnio acid substitution) [37]. The p.S2646F variant (also known as c.7937C>T) is predicted to be tolerated by *in silico* analysis [37]. An aortic CTA measured the aortic diameter at 3.3cm and will be followed with proper longitudinal management and screening.

p.A3542C: An 83-year-old Caucasian-American presented to our practice with a history of a 4.5 cm aortic aneurysm and celiac artery aneurysm with stable dissection. Given these unique findings, clinical genetic testing revealed a heterozygous mutation VUS p.A3542V (poorly conserved position in vertebrates) in ZNF469 with a GS of 64 (similar amnio acid substitution) The p.A3542V variant (also known as c.10625 C>T), is predicted to be tolerated by *in silico* analysis [38].

Subject	Age	Gender	Ethnicity	Family History	Mutation	Location	Zygosity	Vascular Phenotype

1	25	Male	Caucasian	• Marfanoid habitus	C.2193delG	Exon: 1 Codon: 2193	Heterozygous	• Fusiform ectasia of aorta • Bicuspid aortic valve • Marfanoid habitus
2	61	Female	Caucasian	N/A	p.R1875C	Exon: 2 Codon: 1875	Heterozygous	• Spontaneous coronary artery dissection
3	45	Female	Caucasian	• Cerebral aneurysm	p.V271D	Exon: 1 Codon: 271	Heterozygous	• Bilateral vertebral artery dissection
4	72	Female	African	• Aortic aneurysm • Cerebral aneurysm • Aortic Dissection	p.Q3094R p.G2871S p. S2637T	Exon: 2 Codon: 2637, 2871, 3094	Heterozygous	• Bilateral aortoiliac dissections • Aortic aneurysm
5	37	Male	Caucasian	• Aortic aneurysm	p.T910I	Exon: 1 Codon: 910	Heterozygous	• Bicuspid aortic valve

6	76	Female	Caucasian	N/A	p.G1933V	Exon: 1 Codon: 1993	Heterozygous	• Aortic aneurysm
7	56	Female	Asian	• Vertebral and aortic artery aneurysm	p.S2646F	Exon: 2 Codon: 2646	Heterozygous	• Fusiform aortic ectasia
8	83	Male	Caucasian	N/A	p.A3542V	Exon: 2 Codon: 3542	Heterozygous	• Aortic and celiac aneurysm

Table 1. Description of ZNF469 v characteristics and demographics. The patients described in this case series display a variety of vascular phenotypes, ethnic backgrounds, ages, and family health histories. Of the cohort, 5/8 (62.5%) have ectasia/aneurysmal disease, 3/8 (37.5%) have experienced dissections, and 4/8 (50.0%) have a family history of 1 or more first-degree relatives with aneurysmal or dissection disease.

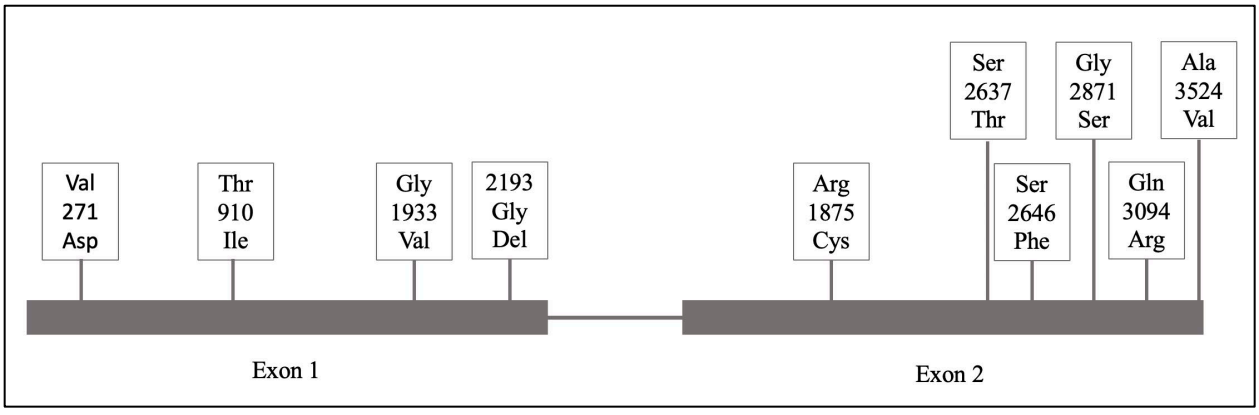

Figure 3. Approximate positions of all mutations across this cohort. The mutations observed in this cohort are confined to coding exons 1 and 2, which are likely to encode proteins with structural alterations.

Discussion

Our discovery of the association of ZNF469 to aneurysmal disease extending to many different arterial circuits through alterations in collagen matrix integrity is novel and impactful. Furthermore, our findings extend beyond one organ system and bring into possible association the human eye and vascular system share many similarities in terms of makeup, consisting of many shared cellular and acellular components, and both are heavily dependent on collagen for structure and function [39]. It seems likely, given these similarities, that a mutation effecting the eye would therefore open the window to vascular dysfunction. In Case 1, the ZNF 469 mutation was pathogenic for brittle cornea syndrome and whilst undergoing a detailed evaluation for ocular pathology, notably has a marfanoid body habitus and fusiform ectasia of his aorta. It may be the case that this mutation opens the door to a new marker for *syndromic disease*, potentially effecting multiple distinct areas of the human body. It should be the goal of future research to connect these associations and determine the true function and impact of ZNF469 and its myriad of mutation's effect on our anatomy and physiology.

The limitations of this study include presence of potential confounding predisposing factors for TAAD (such as hypertension), lack of understanding of definitive downstream molecular and structural impacts of individual, or in combination mutations, and an unclear temporal relationship between mutation and phenotype development. Further study will be necessary to correlate these genetic changes with definitive histopathologic findings secondary to alterations in the encoded collagen matrix protein. Though not all patients included have personal history of aneurysm or dissection, given their mutation status, young age, and positive family history we suspect that these patients have a strong predisposition to developing TAAD and thus will undergo serial monitoring as clinically appropriate. In Subject 7, given her middle age, we believe that her mutation is likely non-pathogenic and are looking forward to genotype assessment of her extended family. However, this finding strengthens the well accepted understanding in genetic medicine

that not all single nucleotide polymorphisms are pathogenic, and opens thought to post transcriptional, epigenetic, and other factors that may influence the genotype to phenotype relationship.

Future Investigations

As we strengthen this association through familial proband genotyping, we hope to define ZNF469 as a causal, pathogenic gene for non-syndromic vascular aneurysmal disease. We plan to genotype the family cohorts associated with each patient to strengthen the association of this genotype to phenotype to prompt further confirmatory studies that may help define this as a causal genetic vasculopathy. This will impact the algorithm for aortopathy screening, which should lead to earlier clinical action in patients with ZNF469 mutations with vasculopathy, potentially mitigating risks of morbidity and mortality.

Conclusion

Our prior case report and the present case series expands the association of ZNF469, with its known effects on in ECM structure and function, and vascular aneurysmal disease. This apparent association, given strong personal and family histories for aneurysmal and dissection disease, gives credence to the notion that ZNF469 encodes a protein that may play a pivotal role in ECM regulation, whether that be direct or indirect, through other proteins such as varying collagens, thrombospondin, or clusterin. A discovery such as this warrants further investigation and may change the way we think about screening patients who have strong personal and family histories of such pathologies to better guide prevention measures, genetic counseling, and management.

Compliance

Due to the nature of the study, IRB approval was obtained (TCU IRB#2022-106), and all patients interviewed provided written consent.

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