MYLK pathogenic variants aortic disease presentation, pregnancy risk, and characterization of pathogenic missense variants

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Purpose: Heritable thoracic aortic disease can result from null variants in MYLK, which encodes myosin light-chain kinase (MLCK). Data on which MYLK missense variants are pathogenic and information to guide aortic disease management are limited.

Methods: Clinical data from 60 cases with MYLK pathogenic variants were analyzed (five null and two missense variants), and the effect of missense variants on kinase activity was assessed.

Results: Twenty-three individuals (39%) experienced an aortic event (defined as aneurysm repair or dissection); the majority of these events (87%) were aortic dissections. Aortic diameters were minimally enlarged at the time of dissection in many cases. Time-to-aortic-event curves showed that missense pathogenic variant (PV) carriers have earlier-onset aortic events than null PV carriers. An MYLK missense variant segregated with aortic disease over five generations but decreases MYLK kinase activity marginally. Functional Assays fail to identify all pathogenic variants in MYLK.

Conclusion: These data further define the aortic phenotype associated with MYLK pathogenic variants. Given minimal aortic enlargement before dissection, an alternative approach to guide the timing of aortic repair is proposed based on the probability of a dissection at a given age.

Key Words: acute aortic dissection; hereditary thoracic aortic disease; MYLK; myosin light-chain kinase; thoracic aortic surgery

INTRODUCTION
Investigation of the genetic basis of thoracic aortic aneurysms and dissections has yielded an increased understanding of the underlying genetic changes that are associated with significantly increased risk. Much of the current literature describes syndromic causes of heritable thoracic aortic disease (HTAD), including Marfan syndrome, Loeys–Dietz syndrome and vascular Ehlers–Danlos syndrome. In recent years, pathogenic variants (PV) of HTAD genes that cause aortic disease with no other syndromic findings have been identified, such as ACTA2 PV, which are responsible for up to 20% of families with non-syndromic HTAD.3,4 and PV of MYLK, PRKG1, and MYH11.3,5

MYLK encodes the Ca2+/calmodulin (CaM)-dependent myosin light-chain kinase (MLCK), which phosphorylates the regulatory light chain to initiate contraction in smooth muscle cells. Using a candidate gene approach, MYLK pathogenic variants were identified in non-syndromic HTAD families. Segregation analyses confirmed that variants leading to MYLK haploinsufficiency caused HTAD, and functional analyses indicated that a missense variant in the CaM-binding domain decreased MLCK kinase activity by 85% and therefore probably caused disease.4 Further support that haploinsufficiency could cause disease came from studies of an inducible, smooth-muscle-specific Mylk knock-out mouse model.6 In the aorta, knock-out of half of the MLCK led to 40% inhibition of regulatory light-chain phosphorylation and aortic contractile response. These results indicate that MLCK levels limit contraction of aortic smooth muscle cells and therefore provide insight into why haploinsufficiency of MYLK leads to thoracic aortic disease.

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More recently, case reports have described individuals and families with pathogenic variants (PV) in MYLK segregating with aortic disease.\textsuperscript{8–9} Aortic measurements in PV carriers before dissection were within normal limits in almost all individuals. In two cases with type B dissections, hypertension was noted, as were the presence of features associated with syndromic causes of thoracic aortic aneurysms and dissections. However, due to the limited number of affected individuals, the clinical phenotype associated with MYLK PV has not been clearly defined. With the addition of MYLK to commercial genetic testing panels, the detection of individuals with PV is becoming more frequent. However, the lack of phenotypic data poses a challenge to clinicians when determining aortic surveillance and the timing of surgical repair of the aorta. The aims of this report are to provide additional information on the thoracic aortic disease phenotype associated with MYLK PV provide gene-specific management recommendations to guide clinical care, and present functional data for new MYLK missense variants.

**MATERIALS AND METHODS**

This study was approved by the institutional review board at the University of Texas Health Science Center in Houston. De-identified data were provided by other centers following institutional review board approval. Informed consent was obtained from all study participants. MYLK variants were identified in our research facility (two families), through diagnostic laboratories (four families), and from published reports.\textsuperscript{8} Sixty individuals from seven unrelated families were studied. Cases who tested positive for a pathogenic or likely pathogenic variant, obligate carriers, and family members who are affected with aortic disease with a 50% chance of carrying the variant were included in the analysis.

Phenotypic data, including demographics, diagnosis of aortic events (defined as aortic dissection or surgical repair of an aortic aneurysm), age at the event, and aortic measurements before or at the time of the event, were collected from medical records. Aortic measurements were taken at large, academic institutions and provided by cardiologists. Aortic dissections were classified as type A or type B using the Stanford criteria. Type A dissections originate in the ascending thoracic aorta, whereas type B dissections originate in the descending aorta. Individuals were classified as having “sudden death” if they died suddenly before hospitalization and no autopsy was conducted. These individuals were considered to have an unknown phenotype and were not included in risk estimates. Previously reported families were contacted to update phenotypic data. Collaborators from outside the United States provided clinical data using a standard data collection form. Church parish records were used to confirm the family history and age of death for the family famIT-001.

Families with the MYLK (NM_053025.3) variants p.Arg1480* (c.4438 C>T), p.Ser1091* (c.3272_3273delCA), and p.Ser1759Pro (c.5275 T>C) have previously been described.\textsuperscript{3,8} Two variants identified by commercial genetic testing laboratories—p Thr1096Glnfs*38 (c.3285delG) and p.Glu1066Asnfs*10 (c.3196delG)—are frameshifts and lead to a premature stop codon and haploinsufficiency. A gross deletion of coding exon 18 to the 3’ untranslated region end of MYLK, also identified by a commercial testing laboratory, is predicted to cause haploinsufficiency. The MYLK p. Tyr1575His (c.4723 T>C) missense variant was identified by exome sequencing and confirmed with Sanger sequencing in a research laboratory using previously described methods.\textsuperscript{9}

**MYLK kinase activity assay**

Three missense variants in MYLK were assessed for functional effects: p.Gly1317Cys (c.3949 G>T), p.Cys1384Gly (c.4150 T>G), and p.Tyr1575His (c.4723 T>C). Wild-type and mutant MYLK plasmids were constructed using site-directed mutagenesis and the recombinant proteins expressed in transfected cell lines. Mutagenesis and expression of MLCK, along with activity and CaM activation assays were performed using previously published methods.\textsuperscript{4} The recombinant proteins were purified by affinity chromatography, and the kinase activity ($V_{\text{max}}$) and binding to CaM ($K_{\text{CaM}}$) were determined.

**Statistical analysis**

Statistical analyses were performed using the Stata software program (version 12.1). Categorical variables were presented as counts and percentages. Age was summarized as the median and interquartile range (twenty-fifth and seventy-fifth percentiles). A Wilcoxon rank-sum test was used to compare median ages between groups. The Kaplan–Meier method was used to produce survival curves using the age at the aortic event or last follow-up in the absence of an aortic event to determine the cumulative risk. A log-rank test was used to compare failure functions. Five individuals, considered obligate carriers, died suddenly with no history of aortic disease. These individuals were not included in the analysis.

A logarithm-of-the-odds (LOD) score was calculated for famIT-001, which carries variant p.Tyr1575His, in which there was one generation between the shared common ancestors and the individuals in the pedigree (Figure 1). The individuals in the first two generations were included in the analysis and designated as unknown for phenotype and genotype data. A two-point LOD score was calculated using Superlink Online (http://bioinfo.cs.technion.ac.il/superlink-online/index-working2.shtml)\textsuperscript{10} for the reconstructed pedigree, with an autosomal dominant model with a reduced penetrance of 0.9. The disease frequency was set to 0.0001 and the causal allele frequency was set to 0.00001.

**RESULTS**

We evaluated the aortic disease phenotype associated with seven pathogenic MYLK variants identified in unrelated families. Two pathogenic variants—p.Arg1480* (c.4438 C>T) and p.Ser1759Pro—were previously described in TAA400 and TAA026, respectively, and showed segregation with disease in family members.\textsuperscript{4} Since publication, one individual in TAA026—a 33-year-old male known to carry the p.
Ser1759Pro variant—underwent prophylactic repair for an ascending aortic aneurysm. Previously published functional assays of the p.Ser1759Pro variant demonstrated an 85% loss of MLCK activity.

In addition to the published families, five pathogenic or likely pathogenic variants from additional families were included in this study (Figure 1a). The first variant, p.Tyr1575His, was detected in a large Italian family using exome sequencing (famIT-001; Figure 1b); no other pathogenic variants were detected in other HTAD genes. This variant was not found in the Exome Aggregation Consortium database, has a combined annotation-dependent depletion score of 24.3, is highly conserved, and is predicted to be damaging by PolyPhen2. The variant was detected in four affected family members and one young, unaffected male, and segregated through distant branches of the family with a LOD score of 4.0. The relationship between the two arms of the family could be traced through church parish records, with one generation between the proband’s generation and the shared common ancestors. Three individuals who died suddenly of unknown causes were obligate carriers. Affected individuals presented with type A aortic dissections at 26 to 77 years of age.

MYLK variant p.Thr1096Glnfs*38 was detected in TAA795 and predicted to lead to nonsense-mediated decay and haploinsufficiency. The variant was detected after the proband experienced a type B dissection at the age of 56 and underwent thoracic endovascular aneurysm repair. On day 5 after the operation, he experienced a retrograde type A dissection and succumbed to complications related to his dissection. Before his retrograde dissection, his ascending aorta measured 3.7 cm (z-score = 0.98). At the time of surgery, his aortic tissue was noted to be thin and friable. There were no other affected family members. Eight family members, aged 25–62 years, also carry the variant. Four of them have undergone imaging and no aortic enlargement was noted.

Fig. 1 Pathogenic MYLK variants and segregation of variant p.Tyr1575His in famIT-001. a Schematic of the long and short form of myosin light-chain kinase (MLCK) showing the location of the MYLK pathogenic variants included in this study. All variants are located in the short form of MLCK, which is the only form expressed in the thoracic aorta. Carriers of the variants depicted here were included in the phenotypic analysis. b Pedigree depicting a multigenerational family with two distant arms affected by thoracic aortic disease. Church parish records were used to confirm common ancestry. Circles represent females and squares represent males. A line through the individual indicates that they are deceased and the legend provides information about the phenotypes associated with the shaded symbols. T/C is used to denote the pathogenic variant genotype and T/T is used to denote the wild-type genotype. b. born; CaM calmodulin; d. died; Ig an immunoglobulin-like domain; Fn Fibronectin-type domain.
A genomic deletion from coding exon 18 to the 3’ untranslated region, leading to haploinsufficiency, was detected in a 46-year-old female who presented with a type A dissection. At the time of dissection, the patient’s ascending aorta measured 4.7 cm and the aortic root measured 3.3 cm. Two additional family members, aged 12 and 73 years, have the variant, but have not had aortic events (defined as aortic dissection or surgical repair of an aortic aneurysm). The 12-year-old has had normal imaging of his entire aorta and the 73-year-old has not had imaging.

Additional data from a published family with a MYLK null variant (p.Ser1091*) were obtained, including current status (history of the aortic event versus no aortic event), age at the aortic event or last follow-up without an event, and thoracic aortic diameters.6 The variant segregated with aortic events in five affected individuals, all of whom presented with type A dissections from 23 to 75 years of age; three resulted in death. Ascending aortic diameters at the time of dissection for the two surviving individuals were 4.7 and 4.8 cm. A sixth individual in this family, who had the variant, chose to undergo prophylactic repair of his aorta at the age of 64 years after learning that his ascending aorta measured 4.1 cm.

Finally, an individual from famFRA-003 with a null variant (p.Glu1066Asnf*10) was included. This is a 45-year-old female who presented with a type A dissection extending to her iliac bifurcation. At the time of the dissection, her aortic root measured 3.2 cm x 3.4 cm and the ascending aorta measured 3.9 cm. She had no family history of aortic dissection or vascular disease.

Two additional variants of uncertain significance were identified in French individuals—p.Gly1317Cys (famFRA-001) and p.Cys1384Gly (famFRA-002). The variants are in the kinase domain of the protein, are highly conserved, and are predicted to be damaging by PolyPhen, and have combined annotation-dependent depletion scores of 23 and 34, respectively. These variants had insufficient data to support pathogenicity (see enzymatic assay results below); therefore, the clinical data on these families were not included in the phenotypic analyses.

### Analyses of aortic disease presentation in individuals with MYLK pathogenic variants

Only individuals with MYLK pathogenic variants were included in these analyses (Table 1). Nearly half were female (n = 27) and all were of European descent. The mean age of the study population still living was 49 years (SD: 21). The majority of study participants had variants leading to haploinsufficiency.

Twenty-three individuals (38%) had experienced an aortic event, defined as aortic dissection or thoracic aortic repair (Table 2). The median age at aortic event was 48 years (interquartile range (IQR) = 42–70). Of these aortic events, 87% (20/23) were thoracic aortic dissections. Type A dissections were the most common (85%; 17/20), followed by type B dissection (15%; 3/20). Surgical repair of an aortic aneurysm was the presenting aortic event in 13% of individuals (3/23). The median age at the time of type A dissection was 48 years (IQR = 44–70).

Aortic measurements (aortic root and/or ascending aorta) were available for 12 participants who experienced aortic events (Supplementary Tables S1 and S2 online). In two individuals, ascending aortic measurements taken before the aortic event were 3.9 and 5 cm, respectively, with no mention of aortic root dilatation. Relevant to these data is the fact that studies indicate that the ascending aorta enlarges with a type A dissection, whereas the aortic root does not.11 Aortic root measurements at the time of dissection were normal in 2 cases...
(3.2 and 3.3 cm), but enlarged in 1 case (5.2 cm). Thus, the median aortic root diameter at the time of type A dissection was 3.3 cm (IQR = 2.0). The ascending aortic measurements in 5 individuals at the time of dissection were between 3.8 and 4.8 cm. One individual was noted to have progressive enlargement of the aortic root after repair of the ascending aortic dissection, and he underwent repair when it reached 4.8 cm. The median ascending aortic diameter in type A dissection was 4.25 cm (IQR = 4.8 cm. The median ascending aortic diameter in type A dissection was 4.25 cm (IQR = 0.8). Of the participants with no history of aortic events, 15 had aortic root and ascending aortic measurements available, and only one had an enlarged aorta: a 77-year-old male null variant carrier with a 4.3 cm aortic root and 3.7 cm ascending aorta (Supplementary Table S2 online). Two of the individuals with type B dissections experienced complications—specifically, paralysis and retrograde type A dissection—but had no aortic root or ascending enlargement. Information on hypertension was available for 10 individuals with aortic dissections, and 50% of participants with hypertension were diagnosed before their aortic event. Persons with hypertension had aortic events, 1 woman, a 26-year-old, had an aortic dissection associated with pregnancy (postpartum).

To determine whether the missense variants disrupted MLCK activity or the activation of kinase activity with CaM binding, assays were completed on p.Tyr1575His, p.Gly1317Cys, and p.Cys1384Gly (Figure 3). Surprisingly, p.Tyr1575His in famIT-001 showed only a 2% decrease in kinase activity compared with wild-type kinase (P = 0.0004; Figure 3a). The p.Gly1317Cys mutant showed a 10% decrease in kinase activity or the activation of kinase activity with CaM binding.

Fig. 2 Kaplan–Meier survival analysis. a Kaplan–Meier failure function evaluating cumulative risk of an aortic event in persons with variants in MYLK. Individuals who died suddenly with no history of an aortic event were not included in the analysis. b Kaplan–Meier curve demonstrating the significant difference in age of onset among individuals with missense variants versus null variants. Persons with missense variants in MYLK presented approximately ten years earlier than those with null variants. c Kaplan–Meier failure function evaluating the cumulative risk of type A aortic dissection in persons with MYLK variants. d Kaplan–Meier curve demonstrating a significant difference in the age of onset for type A dissection in individuals with missense versus null variants. CI confidence interval.
activity, and a 40% increase in CaM concentration was required for half-maximal activation (Figure 3b). Variant p. Cys1384Gly only showed a 2% decrease in kinase activity (Figure 3c). Based on these in vitro assays, none of these variants would be expected to decrease enzymatic activity to the extent of a previously reported PV (p.Ser1759Pro). Therefore, we have classified only p.Tyr1575His as pathogenic based on segregation of the variant with aortic disease in the family, rather than disruption of MLCK enzymatic function.

**DISCUSSION**

Individuals with MYLK PV present a unique challenge in terms of aortic disease management because a subset have minimal enlargement of the aortic root or ascending aorta at the time of a type A aortic dissection. Based on the data presented here, both the root and ascending aorta can enlarge in individuals with MYLK pathogenic variants. Therefore, imaging of the ascending aorta and aortic root should be part of disease management, and both segments of the aorta should be repaired at the time of aortic surgery, even if the segment is not enlarged. After repair of a dissected ascending aorta, the aortic root can enlarge; therefore, imaging should be done to monitor the size of the aortic root. This study also determined that missense variants lead to higher risk and are likely to have an earlier onset of aortic events—specifically type A dissections—compared with null variants. Interestingly, this finding is similar to COL3A1 variants in individuals with vascular Ehlers–Danlos syndrome; null PV carriers tend to present with a milder phenotype and vascular disease onset 10–15 years later than missense PV carriers. MYLK PV carriers have a later onset and lower penetrance of aortic disease compared with other HTAD genes, such as TGFBR1 and TGFBR2.

Given that there may be no aortic enlargement before type A dissections, surgical intervention cannot be based solely on the diameter of the aorta. As more data become available, we propose adapting an alternative surgical timing model for cases with PV in the MYLK gene, similar to that used for hereditary cancer populations. In individuals with BRCA1 or BRCA2 PV surgical recommendations are determined by the age at which the risk for breast or ovarian cancer increases significantly. For example, guidelines from the National Comprehensive Cancer Network recommend that women with PV consider prophylactic bilateral salpingo-oophorectomy surgery between the ages of 35 and 40 years, or older if they have not finished having children, to reduce the risk of ovarian cancer. Similarly, individuals with PV in MYLK should consider surgery based on the age at which the risk for dissection increases significantly, and not solely based on the aortic diameter. Based on our data, individuals with haploinsufficiency variants are at minimal risk for a type A dissection before the age of 40 years; data are limited for missense variants, but the onset of type A dissection is earlier in these cases. Physicians and surgeons should consider discussing with their patients prophylactic surgical repair of the ascending aorta and aortic root to prevent dissections based on both the underlying PV and the age at which the risk for dissection increases. Given the variability of surgical outcomes between institutions, we recommend that carriers of
pathogenic variants in MYLK consider surgery at a tertiary care center with experience in the surgical management of hereditary aortic disease to minimize the risk of unfavorable outcomes. Blood pressure should be well controlled and treatment with β-adrenergic blocking agents should be considered even in the absence of hypertension.

The MYLK p.Tyr1575His variant segregates with disease in distantly related branches of famIT-001 with a LOD score of 4. It is absent in the Exome Aggregation Consortium database and is therefore classified as a pathogenic variant. It was surprising that functional assays of this variant showed only a minimal decrease in kinase activity, most likely not sufficient to cause disease. These findings emphasize the fact that established functional assays do not always correlate with the pathogenicity of variants. In the long form of MLCK, which is responsible for regulatory light-chain phosphorylation in nonmuscle cells, the cellular localization and regulation of MLCK is dependent on phosphorylation of tyrosines in the unique amino-terminal domain of the protein. Therefore, future studies to determine how MYLK p.Tyr1575His predisposes to disease should focus on whether phosphorylation of this tyrosine plays a role in kinase activation in smooth muscle cells. Finally, the difference in the Kaplan–Meier time to aortic event curves between missense and haploinsufficiency MYLK variants raises the possibility that missense MYLK pathogenic variants lead to a dominant negative effect that disrupts protein function to a greater degree than null variants. Further functional analyses of co-expressed wild-type and mutant MLCK proteins will be necessary to determine whether missense variants can disrupt the function of the wild-type protein.

Type B aortic dissection causes life-threatening complications in 25% of cases, including malperfusion leading to paraplegia, periaortic hematoma, and hemorrhagic pleural effusion. Interestingly, among this study population of cases with MYLK pathogenic variants, all individuals with type B dissections experienced life-threatening complications, including retrograde dissection with thoracic endovascular aneurysm repair placement and rupture of the descending aorta. It is also interesting to note that two individuals undergoing surgery of the descending aorta were noted to have fragile aortic tissue.

Importantly, these recommendations are based on data from a small population of PV carriers and represent patients who presented for genetic evaluation due to early onset of aortic dissection or a family history of aortic disease. Thus, these cases may reflect the more severe end of the disease spectrum in carriers of MYLK variants. Skewing of early data on the phenotype associated with novel genes toward more severe phenotypes occurs, including in the early publications on disease presentation in patients with PV in TGFBR1, TGFBR2, BRCA1, and BRCA2. However, the information provided by these cases provides valuable insight into the presentation of the disease and can be used to inform decision-making in terms of the timing of aortic surgical repair.

In summary, these data define the aortic disease presentation associated with MYLK PV both in terms of the aortic disease risk associated with different types of PV and the associated aortic presentations and complications. Our data suggest that the size of the aorta may not be a useful marker of risk of dissection since many of the individuals in this study had dissection at small aortic diameters. Additional markers of aortic instability should be considered in decisions concerning the timing of aortic repair, including the probability of aortic dissections based on the patient’s age and type of PV. Studies involving a larger cohort of patients with MYLK variants are needed to improve the precision of these risk estimates and confirm earlier dissections for all missense pathogenic variants. Furthermore, the present data indicate that additional studies are needed to determine why MYLK heterozygous missense variants with essentially normal kinase activity can nevertheless cause disease.

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