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Genetic Complexity of Mitral Valve Prolapse Revealed by Clinical and Genetic Evaluation of a Large Family

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Abstract

A genetic component to familial mitral valve prolapse has been proposed for decades. Despite this, very few genes have been linked to MVP. Here we describe a four-generation pedigree with numerous individuals affected with severe MVP, some at strikingly young ages. Detailed clinical evaluation was performed on all the affected family members, which demonstrates a spectrum of MVP morphologies and associated phenotypes. Linkage analysis failed to identify strong candidate loci, but revealed significant regions, which we investigated further using whole exome sequencing of one of the severely affected family members. Whole exome sequencing identified variants in this individual that fell within linkage analysis peak regions, but none were obvious pathogenic candidates. Follow-up segregation analysis of all exome-identified variants was done to genotype other affected and unaffected individuals in the family, but no variants emerged as clear pathogenic candidates. Two notable variants of uncertain significance in candidate genes were identified: p.I1013S in *PTPRJ* at 11p11.2 and *FLYWCH1* p.R540Q at 16p13.3. Neither gene has been previously linked to MVP in humans, although *PTPRJ* mutant mice display defects in endocardial cushions, which give rise to the cardiac valves. We detected *PTPRJ* and *FLYWCH1* expression in adult human mitral valve cells, and *in-silico* analysis of these variants suggests they

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may be deleterious. However, neither variant segregated completely with all of the affected individuals in the family, particularly when “affected” was broadly defined. Thus, while we cannot exclude a contributory role for PTPRJ and FLYWCH1 in this family, our study underscores the difficulty involved in uncovering the genomic contribution to MVP, even in apparently Mendelian families.

Introduction

Idiopathic mitral valve prolapse (MVP) is a common valvular condition, with a prevalence of approximately 2–3 percent.[1] Although the prognosis is often benign, MVP can be associated with serious complications, including mitral regurgitation, infective endocarditis and arrhythmias such as atrial fibrillation. Severe mitral regurgitation due to MVP can cause heart failure and requires surgical treatment.[2, 3] While MVP is more commonly sporadic, familial forms have been documented clinically for decades, and include syndromic and non-syndromic forms.[4] Familial MVP is typically inherited as an apparently autosomal dominant trait with reduced penetrance and variable expressivity, and reportedly exhibits locus heterogeneity, although the exact genes involved have not been identified.[5–7]

To date, three different chromosomal loci have been linked to familial MVP [8,9,10] and additionally; the *FLNA* locus has been found to segregate with a rare, X-linked form of valvular heart disease often affecting both mitral and aortic valves,[11, 12] and more recently, *DCHS1* at 11p15.4 has been identified as an MVP disease gene.[13] Individuals affected with MVP may present with co-morbidities such as dilated aortic root (DAR), as seen in some heritable connective tissue disorders, [13–15] although the full spectrum of cardiovascular phenotypes seen in MVP-affected families is unclear. Few studies have interrogated the genetics of MVP using newer unbiased, next-generation sequencing technologies.

In this study, we delineate the spectrum of MVP-associated features that can be associated with familial MVP, and demonstrate the difficulty in unraveling the genetics of the condition.

Materials and Methods

Clinical evaluation and echocardiography

With full informed consent and approval of the UNC Institutional Review Board, family members (7 male subjects and 14 female subjects; age 3 to 95 years) underwent a focused history and physical examination by a cardiologist (C.P. or T.P.), a 12-lead EKG, and standard two-dimensional (2-D) color Doppler echocardiography. All echocardiograms were interpreted in a blinded fashion by two cardiologists with board certification in echocardiography (T.P. and B.C.J.). MVP was diagnosed by 2-D echocardiography using pre-specified diagnostic criteria, including displacement of the leaflet edges, thickness and redundancy of the valve, and the diameter of the mitral annulus. The displacement of each leaflet was measured in the parasternal long-axis view above a line connecting the mid-portions of the annular hinge points.

The thickness of the mitral valve was measured with M-mode recording. Mitral regurgitation was evaluated qualitatively using color-flow Doppler. Subjects were classified according to the following standard criteria:

Definitive mitral valve prolapse: a redundant valve with leaflets >5 mm thick, a total leaflet displacement >2 mm and no evidence of annular dilation.

Equivocal mitral valve prolapse: valve thickness of 4–5 mm or total leaflet displacement of < 2 mm.

Unaffected individuals: valve thickness <4 mm and no leaflet displacement.

Genetic and Molecular Analysis

Genomic DNA was isolated from 10 mL of whole blood using PureGene chemistry. Sample quality and quantity were measured using an Agilent Bioanalyzer. For linkage analysis, 1 mcg of genomic DNA was run on an Illumina Human CytoSNP-12 bead chip. Data from unrelated Caucasian individuals with Western European ancestry (CEU) included in the HapMap project Phase II release (www.hapmap.org) were used to estimate allele frequencies for all SNPs on the Illumina bead chip. Variants included in the linkage analyses were removed if they were estimated to be in modest-to-high linkage disequilibrium (LD) (r^2<0.1) with one other. A final panel of 6,283 independent single-nucleotide autosomal and Chromosome X diallelic variants with estimated minor allele frequency > 0.2 were included in the linkage panel. The software MERLIN was used to perform parametric linkage analyses for two alternate models, both assuming a dominant inheritance model with an uncommon susceptibility allele (assumed frequency = 0.01). Both models allowed for some phenocopies (e.g. observed trait could be due to other genetic or non-genetic factors independent of the currently tested locus). Model 1 coded subjects with equivocal and definitive MVP, as well as DAR as “affected”. Model 2 was stricter and only included those with definitive MVP as affected. IV:12 was affected with MVP per external echocardiogram report and was included as affected in the Model 1 but was labeled as “unknown” in the more conservative Model 2. To reduce the impact of disease misclassification, subjects with missing phenotypes as well as those affected with related phenotypes (e.g. DAR or equivocal MVP for Model 2) had their disease status coded as unknown.

Molecular barcoding, whole exome sequencing (WES) capture, and library preparation on V:2 (Figure 1) was carried out using the Illumina SureSelect All Exon Kit version 4 according to manufacturer’s guidelines. Sequencing, mapping, alignment, and variant calling were done as previously described [16].

Segregation analysis of variants was done via Sanger sequencing of purified PCR products using gene-specific primers (sequences available upon request).

Candidate gene lists were compiled using ontology-based databases (KEGG, release 69.0 and AmiGO, version 1.8) and the STRING database to identify genes related to *FLNA* or *FBN1,* the ECM, or the TGF pathway[17,18]

Human Valve Cell Primary Culture and RT-PCR

Human mitral and aortic valve interstitial cells were obtained through the UNC Human Heart Tissue Bank. Leaflet tissue was procured at the time of valve replacement surgery with the support of the UNC Institutional Review Board. One million human valve interstitial cells from passage 3 or earlier were harvested for RNA isolation and purification using RNeasy mini kit (Qiagen), and 500 ng RNA was used to generate cDNA using ABI high capacity cDNA reverse transcription kit. cDNA was diluted 1:20, and 1 mL was subsequently used as template to screen for expression of human *FLYWCH1, PTPRJ,* and *COL6A3* relative to *ACTB* (sequences available upon request). Template DNA and gene-specific primers were added to EvaGreen mastermix (BioRad) in 20 mL triplicate reactions run on a BioRad CFX96 Lightcycler.

Results

Pedigree

We studied twenty-two members from four generations of a single family containing multiple individuals with MVP (Figure 1). The most severely affected individual, V:2, presented with MVP and severe mitral regurgitation necessitating mitral valve repair at age five. This individual's family history indicated that many of her family members were also affected with MVP, including her brother, her mother, her maternal grandmother, a maternal great-aunt, and her maternal great-grandmother.

Clinical evaluation

We performed a detailed clinical evaluation of 20 available family members, including a physical examination and an echocardiogram; the echocardiogram for IV:12 was performed at another center. Evaluation of the echocardiograms showed that the severity of MVP varied within this family, as did the presence and severity of mitral regurgitation and aortic dilatation (Figure1; Table 1). Six individuals met our pre-specified diagnostic criteria for definitive MVP, and an additional ten individuals were diagnosed with equivocal MVP. Four individuals with MVP also had dilated aortic root (DAR), and four family members with MVP had atrial fibrillation or flutter documented in the medical history (Afib: IV:2, III:2; Flutter: II:2, and II:3). MVP segregated in an apparently autosomal dominant fashion with variable expressivity.

Linkage analysis

In order to identify candidate chromosomal loci associated with the MVP phenotype in the family, we performed genome-wide linkage analysis on the twenty family members we evaluated clinically. DAR and equivocal MVP morphologies have consistently been associated with MVP, are enriched in MVP families, and may represent an MVP spectrum phenotype of shared genetic etiology.[1, 19] Accordingly, the initial linkage analysis using scheme 1 was broad, and included both equivocal and definitive MVP, as well as DAR. Scheme 2 was stricter and only included those with definitive MVP as affected. In scheme 1, multipoint logarithm of the odds (LOD) scores of 2.2 were obtained at chromosome 2qter, 2.0, 1.9 and 1.9 at three separate locations on chromosome 6, and 2.1 at 16pter (Figure 2a).

In scheme 2, LOD scores of 1.2 were obtained at 21 locations across multiple chromosomes (Figure 2b). Although this was not surprising given that this scheme only included six family members, it nevertheless allowed us to highlight particular regions to use for correlation with our exome results on the severely affected proband. None of the candidate loci from the linkage analysis has been previously associated with MVP, and no obvious MVP candidate genes emerged from any of the linkage peak regions. Therefore, all candidate genomic loci with LOD scores greater than 1.0 were further evaluated by performing whole exome sequencing (WES) on the most severely affected family member, V:2, in order to identify rare variants predicted to be deleterious.

Analysis of COL6A3

In the linkage analysis, an ~2.9 Mb linkage peak (defined by 1-LOD support interval) was identified at chromosome 2 in both phenotypic stratification schemes flanking rs4663726 (LOD of 2.2 in scheme 1; 1.2 in scheme 2), which falls within an intronic region of *COL6A3*, the gene encoding the alpha3 chain of the collagen 6 protein. Out of the group of 26 genes found in the chr.2 linkage peak, *COL6A3* stood out, given consistent observations of altered collagen in myxomatous MVP, and high incidence of MVP in patients with Ehlers-Danlos Syndrome (EDS, MIM 130000) carrying mutations in other collagen genes.[20–23] Six *COL6A3* variants were identified in V:2, but all had an allele frequency over 0.39 in the population. Inspection of the genomic region in close proximity to *COL6A3* identified several DNaseI hypersensitivity sites, a 4kb verified Vista enhancer element, and a FANTOM5 -identified enhancer whose expression is tightly linked with expression of *COL6A3.*[24, 25] The approximately 500bp region in which these identified regulatory regions overlap falls within the chr.2 peak region consistently identified in our linkage analysis, and is highly conserved among vertebrate species. We therefore Sanger sequenced this non-coding region, which we designated RR1, in V:2. We detected rs11677932, a known polymorphism whose allele (A/G) frequencies range between 0.2 and 0.8 in different populations, but did not identify any other variants in this region.

Analysis of Candidate Genes

In going through the whole exome data of the severely affected proband, we specifically analyzed any biologically plausible MVP genes, carefully examining all genomic variants from a list of familial MVP candidate genes that were chosen based on relevant biological context. A total of 1,117 genes were carefully evaluated from five categories previously implicated in MVP pathogenesis: Extracellular matrix (ECM) genes, genes in previously-described familial MVP chromosomal loci, genes related to TGFβ signaling, and genes predicted to interact with the MVP-associated genes *FLNA* and *FBN1.* The intersection between these biologically interesting genes and genes harboring rare variants (MAF <0.005) in the severely affected family member V:2 identified *COL18A1* p.R980H (an ECM gene) and *IQGAP1* p.I1071F (a putative *FLNA*-interacting gene). No candidate variants were found in genes related to TGFβ signaling, genes within previously reported candidate MVP loci, or genes predicted to interact with *FBN1.* In addition, we found no likely pathogenic variants in genes clinically associated with cardiovascular phenotypes, including *ACTA2, CBS, COL3A1, FBN1, FBN2, MYH11,* and *MYLK.*

Expanded analysis of whole exome sequencing data

To broaden our analysis beyond just linkage peak regions or MVP pathway enriched genes, we examined the WES data from V:2 for any deleterious variants in the entire exome. This allowed for an unbiased sweep of any gene harboring a rare variant. Expanded analysis of the entire WES data set for candidate pathogenic variants identified two nonsense variants, *PALLD* p.(E446Ter) on 4q32.3 and *ASPH* p.(R183Ter) on 8q12.1. We confirmed that both the *PALLD* and *ASPH* heterozygous truncating variants were inherited from her affected mother, although neither variant segregated with other affected family members definitively affected with MVP. We identified a total of 23 rare (MAF <0.005 in 1000 Genomes) missense variants that could not be excluded as benign, including genes that fell within some of the peak regions identified by the linkage analysis; eleven of these variants were predicted to be damaging by ConDel (Table 2).

Segregation Analysis

In order to evaluate whether any of the predicted deleterious genomic variants in V:2 were also present in other affected family members, we performed a comprehensive segregation analysis of all the WES-identified candidate variants in the twenty-two family members available for genotyping. We evaluated 15 variants, including the eleven rare, predicted deleterious missense variants that fell within our linkage peak regions; the two rare deleterious missense variants in *COL18A1* and *IQGAP1* present on the candidate gene lists; the *COL6A3* regulatory region polymorphism, RR1; as well as the rare truncating variants in V:2 (Figure 3). Two heterozygous missense variants, *FLYWCH1* p.R540Q on chr. 16p13.3, and *PTPRJ* p.I1013S on chr. 11p11.2, segregated significantly with MVP in the family. Both of these genes fell within regions identified in the genome-wide linkage analysis. The *FLYWCH1* p.R540Q variant was present in all six family members with definitive MVP, was present in eight out of ten individuals with equivocal MVP, and was present in all five individuals with DAR, including IV:5 who is currently unaffected with MVP. The *PTPRJ* p.I1013S variant was present in all six family members with definitive MVP, and was present in two out of ten individuals with equivocal MVP. Thus, the *PTPRJ* p.I1013S variant segregates exclusively with definitive MVP in this family, while the *FLYWCH1* variant segregates with a broader cardiovascular phenotype encompassing definitive MVP, DAR, and to a significant extent, equivocal MVP. Given the nuanced clinical variability in MVP, it is difficult to determine whether individuals currently classified as equivocal share an underlying pathophysiology with those that are definitively affected, and/or whether phenocopies may be present in the family. Regardless, equivocal MVP is not a clinically normal state, and importantly, the *FLYWCH1* and *PTPRJ* variants are not present in any of the unaffected individuals (IV:3, III:7, or V:12; Figure 3).

Candidate MVP variant characteristics and qRT-PCR of Human Valve Cells

In the Exome Aggregation Consortium (ExAC) database of 60,706 individuals, the frequency of the identified *FLYWCH1* and *PTPRJ* variants is less than 0.0001, indicating that these variants are extremely rare in the general population. In addition, the amino acid residues affected are highly conserved in mammals, and are located within described functional domains of the proteins (Figure 4A). The expression of *FLYWCH1* and *PTPRJ*

genes has not previously been reported in human heart. Using RT-PCR, we identified both *FLYWCH1* and *PTPRJ* transcripts in primary cultures of human mitral and aortic valve leaflet cells. Expression of *FLYWCH1*, as well as *COL6A3*, appears higher in mitral valve than aortic valve, while expression of *PTPRJ* does not differ between the two tissue types (Figure 4B). Thus, while no single variant was identified as an obvious pathologic candidate, two rare, predicted damaging variants were identified in genes expressed in the heart valves.

Discussion

Studies of familial MVP have thus far been limited by the analysis of relatively small pedigrees, a lack of systematic diagnostic criteria, and a lack of unbiased comprehensive genetic evaluations. We used a combination of clinical, genomic, and bioinformatics tools to systematically evaluate the genetic contribution within a large multigenerational family affected with MVP (Figure 5). Our results support the idea of clinical and genetic heterogeneity in familial MVP, and underscore its genetic complexity.

We took a comprehensive and unbiased approach in order to evaluate all potentially contributory variants in our WES data from the most severely affected family member. We looked at all variants within any genes known to be associated with MVP clinically; we generated lists of candidate MVP genes based on relevant biological context; and we manually examined all truncating and missense variants with a minor allele frequency (MAF) less than 0.005. We cannot exclude that a more common variant also contributes to the pathogenesis of MVP in this family or in other cases of MVP. We filtered our WES-identified missense variants based on whether they were predicted to be deleterious by ConDel, a computational tool with up to 88% accuracy; thus it is unlikely that it failed to identify a true pathogenic missense variant contributing to MVP in the severely affected family member.[26]

The linkage peak seen at chromosome 2qter, a genomic region including the *COL6A3* gene, is interesting given the proposed role of collagen in the pathology of MVP. [27, 28] Though previous studies have failed to identify mutations in collagen genes in individuals with MVP [29,30], collagen abnormalities have been consistently noted in surgical samples from MVP patients requiring repair. Collagen VI is highly expressed in mouse and human developing and adult mitral valve tissue [31–33], a finding we confirmed. Germline variants that affect *COL6A3* transcript or protein could therefore theoretically affect early development of the valves in a manner that leads to deleterious effects later in life. While our data failed to identify a pathogenic variant within *COL6A3* in the most severely affected individual in this family, we cannot definitively rule out a role for *COL6A3* involvement, including genomic variants that regulate *COL6A3* transcription or post-translational processing.

Two rare, predicted deleterious variants in biologically plausible candidate genes segregated significantly with MVP in this family: *FLYWCH1* p.R540Q, on chromosome 16p13.3 and *PTPRJ* p.I1013S on chromosome 11p11.2. Expression of both genes in cells isolated from human mitral and aortic valve leaflets (Figure 4B) suggests they are expressed in the proper target tissue. It is possible that one, neither, or both variants contribute to MVP, in a digenic or oligogenic model of inheritance. It is likely that genetic modifiers, and potentially other

non-genetic factors, also contribute to the variable expressivity seen in this family. Our analysis demonstrates that a definitive single pathogenic variant may not be uncovered in all apparently genetic cases, even in large affected families where the condition appears to segregate in a Mendelian fashion.

Nevertheless, the rare, predicted damaging variants that we did identify as segregating with MVP in this family, make interesting candidate variants that could be further explored in future studies. *PTPRJ* (also known as CD148 or DEP-1) is a receptor tyrosine phosphatase implicated in regulation of ERK signaling, a pathway known to be important for proper early valve development.[34] The p.I1013S variant in the PTPRJ juxtatransmembrane region (Figure 4A)is near p.K1016A, a variant that has been reported to decrease binding to ERK½ [35], suggesting that one possible functional effect of the p.I1013S variant is that it may alter ligand-binding or signaling important for valve development. Mice homozygous for a null allele of *PTPRJ* have endocardial cushion defects and broad defects in endothelial cell signaling. As the heart valves arise almost entirely from endothelial cells and the endothelial cushions in particular, genetic disruption of *PTPRJ* is a biologically plausible mechanism contributing to development of MVP.[36]

FLYWCH1 is a FLYWCH-type zinc-finger containing protein identified in a recent study as a candidate master regulator of atherogenesis, giving precedent to the idea that *FLYWCH1*-mediated transcriptional regulation may be particularly important for the cardiovascular system.[37] The mature flywch1 protein (Q4VC44, UniProt) is characterized by a series of five FLYWCH zinc finger domains, which can participate in nucleic acid binding and regulation of gene expression.[38, 39] The *FLYWCH1* p.R540Q variant that segregates with MVP in this family resides in the middle of the fourth FLYWCH zinc finger domain, defined by similarity as residues 508–566 in UniProt. Arginine residues, in part because of their acidic charge, are thought to be critical for mediating the DNA-interacting function of many DNA-binding proteins that regulate transcription.[40] Modeling the substitution of arginine for glutamine (hydrophilic and non-charged) at this position shows the variant residue may alter the normal association with the zinc ion, and with DNA at this site (Figure 4C). Thus, exome sequencing identified predicted deleterious variants in two genes, both of which have been reported to play a role in cardiovascular development or function. While genetics may play a role in some MVP families, a strong contribution from a single gene may not define the pathophysiology in all cases.

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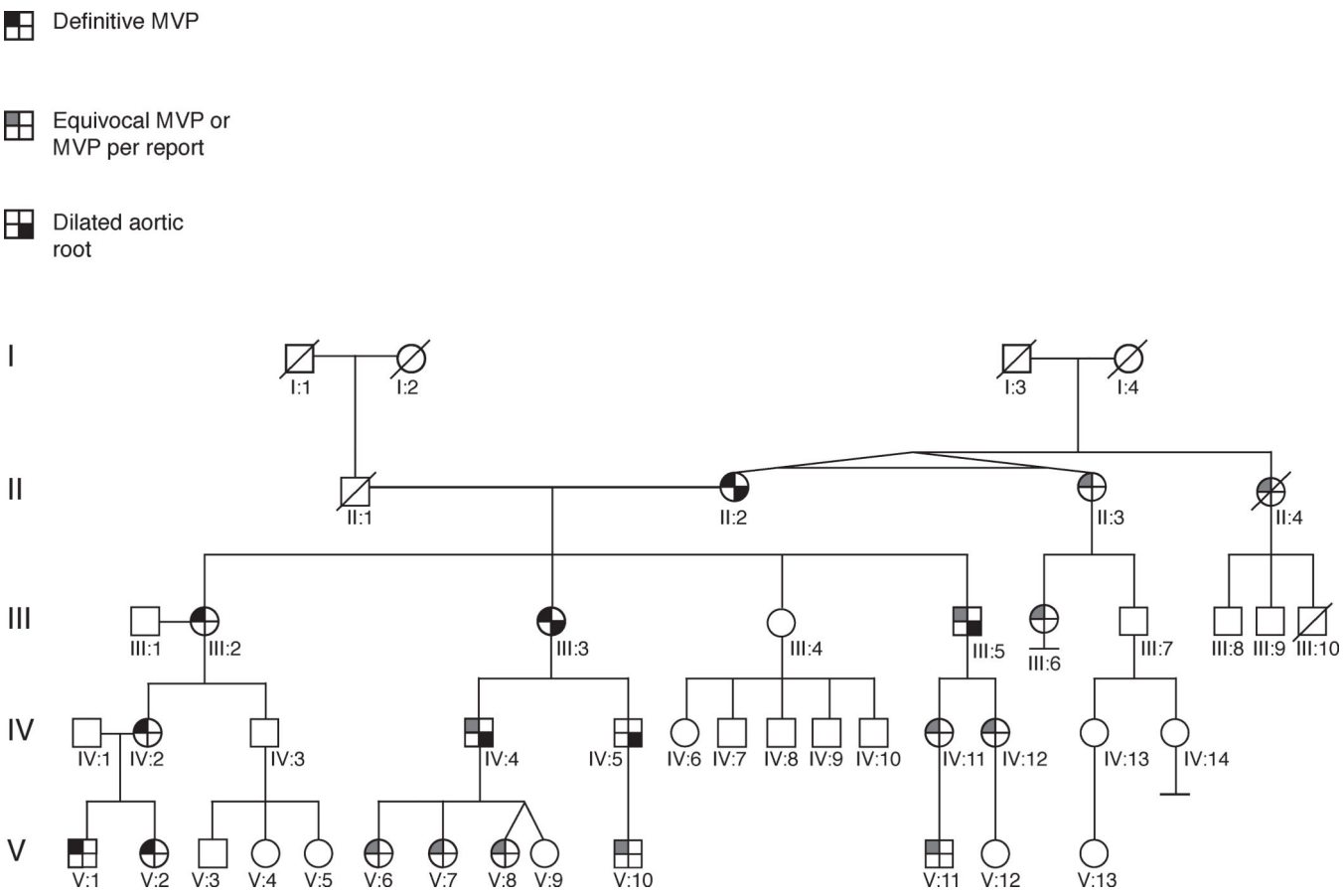


Figure 1:

Pedigree demonstrating apparently autosomal dominant segregation of MVP.

A five-generation family history was obtained during a family research visit in which medical histories could be elicited from members of each of the major branches of the family. The initial self-reported diagnoses of mitral valve prolapse or other cardiovascular phenotypes were subsequently evaluated in more detail by study investigators to define the specific phenotypes (definitive MVP, equivocal MVP, dilated AR) that were present or absent.

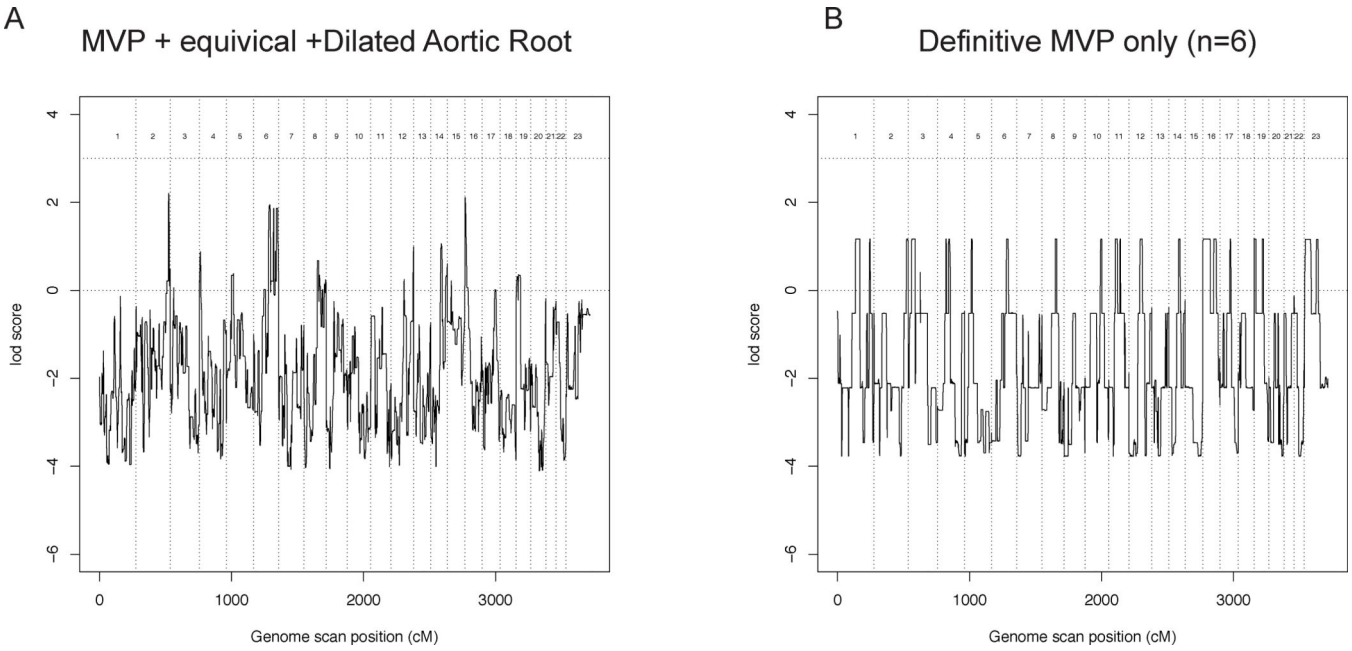


Figure 2:

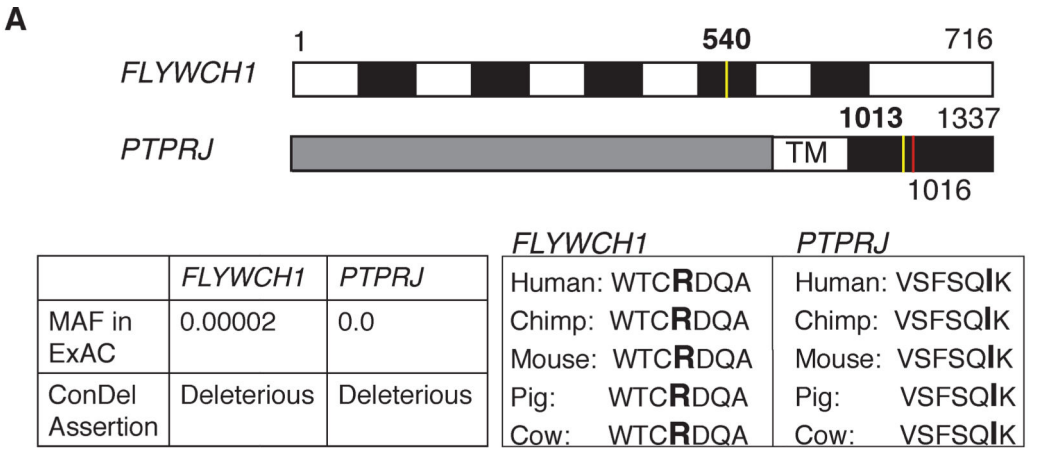
Genome-wide linkage analysis identifies suggestive multipoint LOD scores.

Linkage analysis was performed in the MVP family using the Illumina Human CytoSNP-12 genotyping bead chip assay, with two different schemes for affected status based on the stringency of the echocardiographic findings. There was no definitive evidence for linkage to any particular locus. A.) In the broader scheme 1, suggestive LOD scores were identified at 2q, 6q, and 16p. In the stricter scheme 2, suggestive LOD scores were identified at additional chromosomal loci, including 3p, 8, proximal 11p, and 12.

Family member		V:1	IV:1	IV:2	V:2	III:2	IV:3	V:6	V:7	IV:4	V:8	III:3	IV:5	V:10	II:2	V:11	IV:11	III:5	IV:12	V:12	III:6	II:3	III:7
Phenotype		D	U	D	D	D	U	E	E	E	E	D	U	E	D	E	E	E	R	U	E	E	U
Genes in linkage peak regions																							
Chr.16	FLYWCH1 R540Q	+	-	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	-	-	+	-
Chr.16	MEFV G678E				+	-		(-)	(-)	(-)	(-)	(-)	(-)	(-)	-	(-)	(-)	(-)	(-)	(-)			
Chr.14	TDP1 L255S				+			(-)	(-)	(-)	(-)	-	(-)	(-)	-								
Chr.11	PTPRJ I1013S	+	-	+	+	+	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	+	-
Chr.11	UNC93B1 A71T			-	+	-		(-)	(-)	(-)	(-)	-	(-)	(-)	-	(-)	(-)	(-)	(-)	(-)			
Chr.11	FAT3 A3418S		+	-	+	-		(-)	(-)	(-)	(-)	-	(-)	(-)	-	(-)	(-)	(-)	(-)	(-)			
Chr.8	EXT1 G212A		-	-	+	-		(-)	(-)	(-)	(-)	-	(-)	(-)	-	(-)	(-)	(-)	(-)	(-)			
Chr.8	HEY1 T195M		-	+	+	+		(-)	(-)	(-)	(-)	-	(-)	(-)	-								
Chr.6	ANKRD6 R13H			+	+	-		(-)	(-)	(-)	(-)	-	(-)	(-)	-	(-)	(-)	(-)	(-)	(-)			
Chr.X	DMD R555C				+			(-)	(-)	(-)	(-)	-	(-)	(-)	-								
Chr.11	DDX10		+	-	+	-		(-)	(-)	(-)	(-)	-	(-)	(-)	-	(-)	(-)	(-)	(-)	(-)			
Candidate genes																							
Chr.21	COL18A1 R980H		-	+	+	-	-								-				-	-			
Chr.15	IQGAP1 I1071F		-	+	+	-									-								
Truncating variants in proband and RR1																							
Chr.4	PALLD E446Ter		-	+	+	-									-								
Chr.8	ASPH R183Ter		-	+	+	+									-								
Chr.2	COL6A3 RR1 snp	A/G	G/G	A/A	A/G	A/G	A/A					A/G	A/G	G/G	A/G	A/G			A/G	G/G			A/G

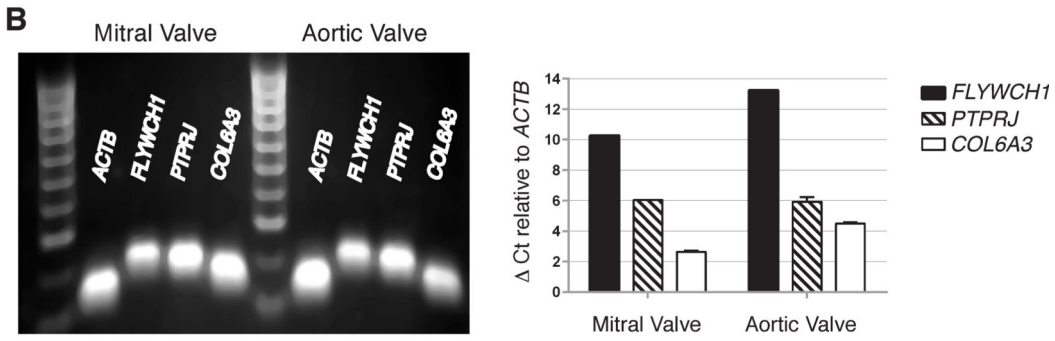
Figure 3:

Segregation analysis of candidate variants in family members available for genotyping. Candidate variants in the WES data from family member V:2 were investigated for co-segregation in affected and unaffected family members using Sanger sequencing. Inferred genotypes are shown in parentheses. Each column corresponds to a single individual from the MVP family pedigree that was available for genotyping. The number at the top of the column refers to the individual as they are labeled in the full pedigree of Figure 1. D=definitive MVP, U=unaffected, E=equivocal MVP, R=MVP per external report. Columns that correspond to the proband's affected mother, grandmother, maternal great-aunt, and great-grandmother are shaded grey. *FLYWCH1* and *PTPRJ* genotypes for informative unaffected individuals are boxed in bold.



	FLYWCH1	PTPRJ
MAF in ExAC	0.00002	0.0
ConDel Assertion	Deleterious	Deleterious

FLYWCH1	PTPRJ
Human: WTC**R**DQA	Human: VSFSQ**I**K
Chimp: WTC**R**DQA	Chimp: VSFSQ**I**K
Mouse: WTC**R**DQA	Mouse: VSFSQ**I**K
Pig: WTC**R**DQA	Pig: VSFSQ**I**K
Cow: WTC**R**DQA	Cow: VSFSQ**I**K



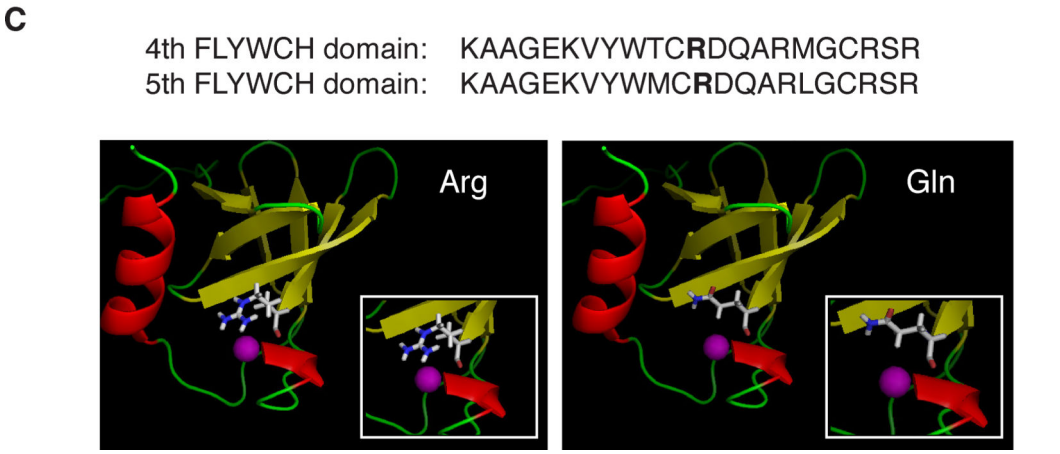


Figure 4:

Variants identified in FLYWCH1 and PTPRJ provide strong candidates for a molecular etiology of MVP in the family.

The FLYWCH1 and PTPRJ variants identified in the MVP family are rare, predicted deleterious, and found in genes expressed in human mitral and aortic valve cells. A. Top: Graphic depiction of human *FLYWCH1*, with FLYWCH zinc-finger domains shown in black, and p.R540Q variant position as yellow vertical line. Graphic depiction of human *PTPRJ* showing p.I1013S residue (yellow line) within catalytic cytoplasmic domain (black),

downstream of the extracellular region (grey) and transmembrane domain (TM), and adjacent to residue 1016 (red line) reported to result in decreased binding to ERK½. *FLYWCH1* p.R540Q and *PTPRJ* p.I1013S variants are conserved among mammals, rare in the general population, and predicted to be damaging. B). RT-PCR results show relative levels of *FLYWCH1*, *PTPRJ*, and *COL6A3* transcripts in cDNA from human mitral and aortic valve normalized to *ACTB*. Delta Ct = number of amplification cycles to reach the threshold when actin Ct is set as zero. Higher positive delta Ct indicates lower levels of expression compared to *ACTB*. Data are presented as mean of three experiments with standard deviation error bars. C). The fourth and fifth FLYWCH domains of *FLYWCH1* are 98% similar. Residues flanking R540 (bold) are shown in the top sequence, and the cognate region of the fifth FLYWCH domain used in the model is shown the bottom sequence. Bottom left panel: PyMol model of protein data bank structure 2rprA, human flywch1 residues 595–674. Residues comprising a helical structure are colored red, beta strands as yellow, and looping regions as green. The arginine occupying the cognate position to residue R540 is depicted as white sticks with hydrogens colored blue, and can be seen in close association to the zinc molecule, shown as a magenta sphere. Inset: Modeled substitution of glutamine at the cognate position 540, normally occupied by arginine. Insets zoom in on the variant – zinc ion region.

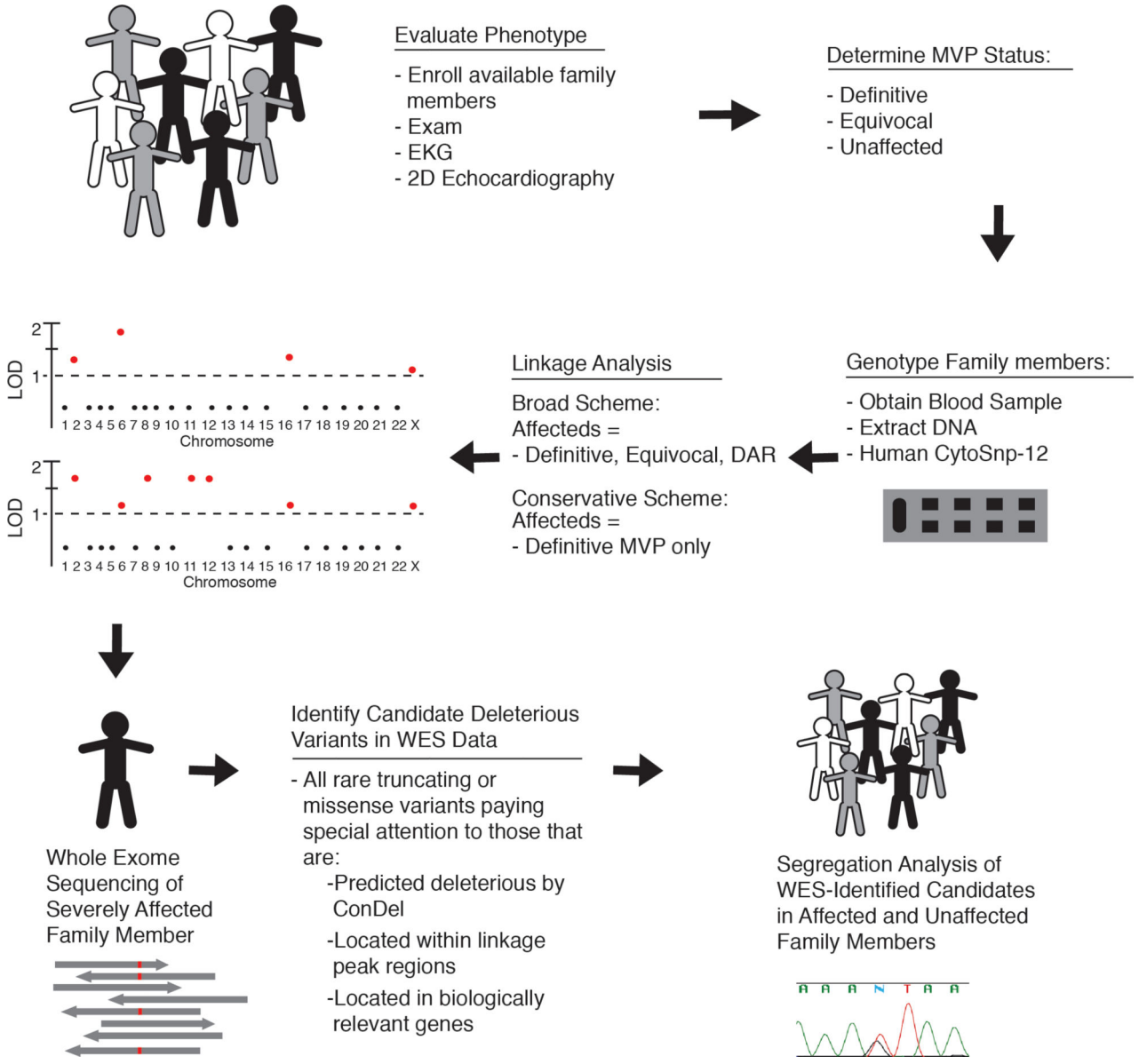


Figure 5:
Clinical and genomic evaluation strategy to identify candidate MVP-associated variants.

Table 1:

Echocardiographic Characteristics of Pedigree Members.

Pedigree ID	Age at echo (years)	Sex	MVP	Mitral Regurgitation	Aortic Root Dilatation
II:2	95	F	Yes	Mild	Yes
II:3	94	F	No	Mild	No
III:2	67	F	Yes	Mild	No
III:3	63	F	Yes	Mild	Yes
III:5	66	M	Equivocal	Trivial	Yes
III:6	66	F	Equivocal	Mild	No
III:7	65	M	No	No	No
IV:2	36	F	Yes	Mild	No
IV:3	46	M	No	No	No
IV:4	42	M	Equivocal	Trivial	Yes
IV:5	44	M	No	No	Yes
IV:11	38	F	Equivocal	Trivial	No
IV:12	33	F	Per Report	Mild	No
V:1	20	M	Yes	Trivial	No
V:2	5	F	Yes	Severe	No
V:6	22	F	Equivocal	Trivial	No
V:7	20	F	Equivocal	No	No
V:8	16	F	Equivocal	No	No
V:10	4	M	Equivocal	No	No
V:11	3	M	Equivocal	No	No
V:12	3	F	No	Trivial	No

Presence and severity of MVP, mitral regurgitation, and dilated aortic root was assessed by double-blind analysis of 2-D echocardiograms and Doppler.

Table 2:

Rare variants in linkage peak regions

Gene	Variant Transcript	Variant Protein	ConDel Prediction	MAF in ExAC
EXT1	NM_000127.2:c.635G>C	NP_000118.2:p.Gly212Ala	deleterious	1.66E-05
HEY1	NM_012258.3:c.854C>T	NP_036390.3:p.Thr285Met	deleterious	0.002054
ANKRD6	NM_014942.4:c.38G>A	NP_055757.3:p.Arg13His	deleterious	1.45E-03
MEFV	NM_000243.2:c.2033G>A	NP_000234.1:p.Gly678Glu	deleterious	2.47E-05
FLYWCH1	NM_032296.2:c.1619G>A	NP_115672.2:p.Arg540Gln	deleterious	2.68E-05
DMD	NM_004010.3:c.1318C>T	NP_004001.1:p.Arg440Cys	deleterious	4.77E-05
UNC93B1	NM_030930.2:c.424G>A	NP_112192.2:p.Ala142Thr	deleterious	7.73E-05
PTPRJ	NM_002843.3:c.3038T>G	NP_002834.3:p.Ile1013Ser	deleterious	0
FAT3	NM_001008781.2:c.10252G>T	NP_001008781.2:p.Ala3418Ser	deleterious	0.001832
DDX10	NM_004398.2:c.1979C>T	NP_004389.2:p.Ser660Phe	deleterious	0.0004275
TDP1	NM_018319.3:c.764T>C	NP_060789.2:p.Leu255Ser	deleterious	3.30E-05
VPS13B	NM_152564.3:c.5606C>T	NP_689777.3:p.Thr1869Met	Neutral	0.0009971
TXNRD1	NM_001093771.1:c.167G>C	NP_001087240.1:p.Arg56Thr	Neutral	0.001549
UHRF1BP1L	NM_015054.1:c.2302C>T	NP_055869.1:p.Leu768Phe	Neutral	8.27E-06
ENPP3	NM_005021.3:c.86T>C	NP_005012.2:p.Leu29Pro	Neutral	0.001522
ARL13A	NM_001162491.1:c.166G>A	NP_001155963.1:p.Glu56Lys	Neutral	0.001208
IRS4	NM_003604.2:c.1315A>T	NP_003595.1:p.Ser439Cys	Neutral	0.001039
KIAA2022	NM_001008537.2:c.133G>A	NP_001008537.1:p.Ala45Thr	Neutral	0.0003268
RPGR	NM_001034853.1:c.3062T>A	NP_001030025.1:p.Val1021Glu	Neutral	9.79E-05
AGAP2	NM_014770.2:c.2414C>T	NP_055585.1:p.Ala805Val	Neutral	0poor coverage
MYO1A	NM_005379.2:c.2624G>A	NP_005370.1:p.Gly875Glu	Neutral	0
D2HGDH	NM_152783.3:c.1039G>A	NP_689996.4:p.Ala347Thr	Neutral	0.0003395