HHS Public Access

Author manuscript

Am J Med Genet A. Author manuscript; available in PMC 2016 September 01.

Published in final edited form as:

Am J Med Genet A. 2015 December ; 167A(12): 2975–2984. doi:10.1002/ajmg.a.37297.

Exome Analysis of a Family with Wolff–Parkinson–White Syndrome Identifies a Novel Disease Locus

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Abstract

Wolff–Parkinson–White (WPW) syndrome is a common cause of supraventricular tachycardia that carries a risk of sudden cardiac death. To date, mutations in only one gene, *PRKAG2*, which encodes the 5’ -AMP-activated protein kinase subunit γ-2, have been identified as causative for WPW. DNA samples from five members of a family with WPW were analyzed by exome sequencing. We applied recently designed prioritization strategies (VAAST/pedigree VAAST) coupled with an ontology-based algorithm (Phevor) that reduced the number of potentially damaging variants to 10: a variant in *KCNE2* previously associated with Long QT syndrome was also identified. Of these 11 variants, only *MYH6* p.E1885K segregated with the WPW phenotype in all affected individuals and was absent in 10 unaffected family members. This variant was predicted to be damaging by in silico methods and is not present in the 1,000 genome and NHLBI exome sequencing project databases. Screening of a replication cohort of 47 unrelated WPW

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Conflicts of interests: none.

SUPPORTING INFORMATION

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patients did not identify other likely causative variants in *PRKAG2* or *MYH6. MYH6* variants have been identified in patients with atrial septal defects, cardiomyopathies, and sick sinus syndrome. Our data highlight the pleiotropic nature of phenotypes associated with defects in this gene.

Keywords

Wolff–Parkinson–White; whole exome sequencing; MYH6

INTRODUCTION

Wolff Parkinson, and White first identified a series of patients with the following features: (i) a bundle branch block; (ii) a short PR interval; and (iii) paroxysms of tachycardia. In 1940, this combination of pre-excitation and paroxysmal tachycardia was termed Wolff–Parkinson–White (WPW) syndrome [Wolff et al., 2006]. WPW is commonly associated with paroxysmal supraventricular tachycardia (1.5–3.1 per 1,000 persons) [Guize et al., 1985] that is maintained by accessory pathway(s) secondary to a developmental cardiac defect in atrioventricular electrical insulation [Kent, 1893]. Although electrophysiological studies remain the gold standard for differentiating various phenotypes, the existence of identical electrophysiological characteristics in a single cohort argues for a common genetic cause. The familial occurrence of the WPW syndrome is well documented, is typically inherited in an autosomal dominant pattern, and is sometimes associated with familial cardiomyopathy [Harnischfeger, 1959; Massumi, 1967; Schneider, 1969]. The molecular genetics of WPW have been investigated but to date only a single gene, *PRKAG2*, has been identified and mutations in this gene explain only a small fraction (<5%) of WPW (Gollob et al., 2001 a,b).

Advances in next generation sequencing modalities, such as whole-exome sequencing (WES), have revolutionized the study of genetic susceptibility to disease. However, in light of the large number of variants identified in these datasets, variant prioritization and identification of disease-causing variants remain a significant challenge. Variant prioritization tools, such as SIFT [Kumar et al., 2009] and PolyPhen2 [Adzhubei et al., 2010] rely on phylogenetic conservation to identify damaging variants and thus are unable to score a large number of variants that reside in non-conserved regions of unknown structure. Moreover, these tools often have low specificity and sensitivity [Flanagan et al., 2010]. The variant annotation, analysis, and search tool (VAAST) utilizes a more accurate and comprehensive approach to variant prioritization, by using the global, genome-wide frequency of observing an amino acid substitution in any gene [Yandell et al., 2011; Hu et al., 2013]. This approach allows VAAST to score any coding change regardless of conservation [Rope et al., 2011; Hu et al., 2013; McElroy et al., 2013; Shirley et al., 2013]. Pedigree-VAAST (pVAAST) [Hu et al., 2014] performs linkage analysis by calculating a novel gene-based LOD score specifically designed for sequence data. The LOD score at each locus is incorporated directly into VAAST to increase the accuracy and greatly decrease the bioinformatic complexity of family-based disease-gene identification efforts. Finally, the phenotype driven variant ontological re-ranking (Phevor) [Singleton et al., 2014] tool integrates knowledge from multiple biomedical ontologies with VAAST output to

reprioritize candidate disease-causing variants. In this report, we combine these novels variant prioritization strategies to identify disease-causing variants in whole exomes from a multi-generation family with WPW.

MATERIALS AND METHODS

Patient Enrollment and Phenotyping

A multigenerational Caucasian family with WPW (K32326) was identified in the Heart Center at Primary Children's Hospital. Clinical evaluation of family members was performed by an electrophysiologist, a medical geneticist, and genetic counselor, who excluded confounding alternatives such as the mitochondrial MELAS syndrome [Aggarwal et al., 2001]. WPW patients were diagnosed using strict electrocardiographic criteria: (i) short PR interval and (ii) presence of a ventricular pre-excitation or delta wave, as determined by an electrophysiologist (SPE, EVS, MT-F).

With University of Utah Institutional Review Board approval, members of the family were enrolled in the University of Utah Pediatric Cardiology Genotype-Phenotype Core after obtaining written informed consent. All human subjects' research were performed in accordance with relevant guidelines and regulations. DNA was isolated from peripheral blood samples using a Centra Autopure LS (Qiagen, Valencia, CA) in the University of Utah Center for Clinical and Translational Science. DNA samples were analyzed by agarose gel electrophoresis to confirm the DNA integrity and quantitated using a Nanodrop.

Analysis of *PRKAG2*

PCR primers were designed to amplify the coding exons, as well as at least 50 nucleotides of the surrounding introns, of *PRKAG2* using the ExonPrimer utility in the UCSC genome browser (http://genome.ucsc.edu/: PCR Primers in Supplemental Table SII) and used to amplify DNA from two of the patients. An aliquot of DNA was analyzed by agarose gel electrophoresis and then the PCR product was purified by treating with 4 μl of Exo-SAP-IT (Affymetrix) at 37°C for 2 hr and 80°C for 15 min. The PCR product was then submitted to the University of Utah DNA sequencing core for analysis and results compared to the published *PRKAG2* sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Exome Sequencing

DNA from five members of K32326 (Fig. 1: I:2, II:2, II:5, III:6, and III:7) were sent to the Baylor Hopkins Center for Mendelian Genomics for WES. In brief, 1 μg of DNA was used to construct an Illumina paired-end pre-capture library according to the manufacturer's protocol (Illumina Multiplexing_SamplePrep_-Guide_1005361_D). The complete protocol and oligonucleotide sequences are accessible from the Baylor Human Genome Sequencing Center (HGSC) website (https://hgsc.bcm.edu/sites/default/files/documents/Illumina_Barcoded_Paired-End_Capture_Library_Preparation.pdf). Four pre-captured libraries were pooled and then hybridized in solution to the HGSC CORE design [Bainbridge et al., 2011] (52Mb, NimbleGen) according to the manufacturer's protocol *NimbleGen SeqCap EZ Exome Library SR User's Guide (Version 2.2)* with minor revisions. The sequencing run was performed in paired-end mode using an Illumina HiSeq 2000

platform, with sequencing-by-synthesis reactions extended for 101 cycles from each end and an additional 7 cycles for the index read. With a sequencing yield of 12 Gb, coverage depth of 20X or greater was achieved for 92% of the targeted exome bases. Illumina sequence analysis was performed using the HGSC Mercury analysis pipeline (https://www.hgsc.bcm.edu/software/mercury) that moves data through various analysis tools from the initial sequence generation on the instrument to annotated variant calls (SNPs and intra-read in/dels). Reads were mapped to the GRCh37 Human reference genome (http://www.ncbi.nlm.nih.gov/projects/genome/assembly/grc/human/) using the Burrows-Wheeler aligner[Li and Durbin, 2009] (BWA, http://bio-bwa.sourceforge.net/) to produce BAM [Li et al., 2009] (bi-] (binary alignment/map) files. Quality recalibration was performed using GATK [DePristo et al., 2011] (http://www.broadinstitute.org/gatk/), and where necessary separate sequence-event BAMs were merged into a single sample-level BAM. Using the software package SAMtools [Li et al., 2009], the aligned sequencing reads were converted and merged into sorted and indexed BAM files. The SAMtools utilities mpileup and bcftools were implemented to call sequence variants. To reduce the number of false positives in the call-set, the five individuals in the family were called together with 139 individuals from the 1,000 Genomes project. ANNOVAR was used to identify variants not previously reported in the 1,000 Genome Project (Phase 1 All-Sites (2011_05)), dbSNP databases [Sherry et al., 2001] (dbSNP build 132), or present with a Minor Allele Frequency (MAF) <0.1% in Caucasians. To predict deleterious effects of non-synonymous amino acid changes, ANNOVAR utilizes various functional annotation algorithms such as SIFT [Kumar et al., 2009], PolyPhen2 [Adzhubei et al., 2010], and MutationTaster [Schwarz et al., 2010]. AlignGVD [Tavtigian et al., 2006] predictions were also made using Alamut software (v2.3: Interactive Biosoftware, Rouen, France).

Further variant prioritization was accomplished through the use of VAAST [Yandell et al., 2011], which combines variant frequency data, mutation severity, and conservation into a single score that is compared genome wide. The analysis was performed following best practices as described in the publication by Kennedy et al. [2014]. Because all of the sequenced individuals are related, pedigree-VAAST (pVAAST) was chosen over standard VAAST analysis [Hu et al., 2014]. pVAAST further empowers the standard VAAST algorithm by probabilistically calculating the degree to which variants follow a specified inheritance pattern. In this case, the disease follows a dominant mode of inheritance with reasonably high penetrance, so the pVAAST analysis was parameterized accordingly. pVAAST results were then re-ranked using Phevor [Singleton et al., 2014]. The Phevor tool takes rankings from gene prioritization tools and re-ranks them based on phenotype information though terms in biomedical ontologies such as GO [Ashburner et al., 2000] and HPO [Kohler et al., 2014]. For this analysis, the Phevor was run using the following HPO terms: prolonged QRS complex (HP:0006677), shortened PR interval (HP:0005165), paroxysmal supraventricular tachycardia (HP:0004763), sudden cardiac death (HP:0001645), ventricular pre-excitation with multiple accessory pathways (HP:0006684), paroxysmal atrial fibrillation (HP:0004757), stroke (HP:0001297), cardiomyopathy (HP:0001638), palpitations (HP:0001962) which describe the WPW phenotype. Cardiac expression of candidate genes was assessed from GenAtlas data accessed by searches of the BioGPS portal (biogps.org).

Validation of Variants Identified by Exome Sequencing

PCR primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to amplify and validate variants detected by Exome Sequencing (PCR primers described in Supplemental Table SIII). The PCR product was purified and sequenced as described above. Once a variant was confirmed all available family members, affected and unaffected, were screened in the same way to assess variant segregation.

Analysis of the Replication Cohort

Forty-seven unrelated patients diagnosed with WPW by ECG and enrolled into the Genotype-Phenotype Core served as a comparison cohort: eight of these patients reported a family history of WPW. Primers were designed to amplify coding exons and exon-intron boundaries of candidate genes using the ExonPrimer utility, as described above (Supplemental Table SII). All patient DNA samples (10 ng per sample) were amplified by PCR in duplicate, along with negative (water) controls, using Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and analyzed on a Lightscanner (Biofire, Salt Lake City, UT), as described previously [Arrington et al., 2008, 2012]. Samples giving abnormal profiles were analyzed by DNA sequencing, as described above.

RESULTS

Clinical Characteristics of Family K32326

The pedigree for K32326 is shown in Figure 1. All the enrolled family members were previously diagnosed with WPW or diagnosed by ECG at the time of enrollment.

Patient I.2 presented to a local emergency department at age 61 years with chest pain and palpitations. The patient's heart rate was 216 bpm and his rhythm was supraventricular tachycardia (SVT) with a probable rate-related bundle branch block. With adenosine administration, the tachycardia terminated transiently but returned within minutes. Ultimately, the patient converted to sinus rhythm with intravenous diltiazem administration. While no delta wave was present when in sinus rhythm, the response to adenosine and family history suggests the presence of a concealed accessory pathway. Subsequently, the patient was diagnosed with aortic stenosis and later, with atrial fibrillation (AF): we were unable to obtain surgical record to determine whether the aortic stenosis was calcific.

Patient II.2 was diagnosed with WPW and a subaortic membrane as a child. At age 19, the patient underwent resection of the subaortic membrane and a septal myectomy. With regard to the subaortic membrane, there was not a muscular component to the membrane itself, rather there was additional septal hypertrophy below the membrane that was felt to be a secondary consequence of the obstructive membrane. An intraoperative electrophysiology study was also performed with ablation of multiple accessory atrioventricular pathways. In the immediate post-operative period, the patient was hemodynamically unstable with ventricular tachycardia and underwent a catheter-based electrophysiology study/ ablative procedure. This procedure was complicated by complete heart block and possible incomplete ablation of ventricular tachycardia necessitating placement of an ICD. The

patient subsequently developed recurrent left-ventricular outflow tract obstruction at age 29 years and underwent repeat subaortic membrane resection and a valve sparing Konno procedure.

Patient II.5 was diagnosed with WPW at age 28 years after presenting with SVT following a routine surgical procedure. The patient's heart was structurally normal with normal ventricular function. A radiofrequency (RF) ablation procedure was performed that identified two left-sided accessory pathways and a right-sided fasciculoventricular pathway, that was not a participant in SVT circuit. The two left-sided accessory pathways were ablated. Shortly thereafter, the patient experienced a syncopal episode and was noted to be in a wide QRS complex tachycardia, consistent with SVT with a rate-related right bundle branch pattern (Fig. 2). Over the next year, the patient underwent two additional RF ablation procedures to ablate the recurrence of the manifest left-sided pathways.

Patient II.6 was diagnosed with WPW as at 5 years of age. This patient experienced multiple episodes of SVT, often with a rate-related right bundle branch pattern. After experiencing three episodes of syncope despite beta-blocker therapy, the patient underwent RF ablation of a left lateral accessory pathway at age 14. The ablation procedure was transiently effective, but subsequent ECGs demonstrated persistent ventricular pre-excitation. The patient's heart is structurally normal with normal ventricular function.

Patient III.7 was found to have ventricular pre-excitation at the time of enrollment in this research project by a screening ECG (Fig. 2). The patient has no history of tachyarrythmia or other cardiac symptoms.

Patient III.8 presented with neonatal SVT and was diagnosed with WPW syndrome (Fig. 2). The rhythm was initially controlled with propranolol, which was discontinued at around 1 year of age. The patient is now 10 years old, still has evidence of ventricular preexcitation on ECG but has not had any further episodes of SVT. The patient's heart is structurally and functionally normal.

Screening members of this family by PCR and DNA sequencing failed to identify any disease-causing variants in *PRKAG2*. Therefore, this kindred was considered an excellent candidate family for whole exome sequencing for the identification of novel disease associated loci.

Exome Sequence Analysis and Segregation of Variants

DNA from five family members I:2, II:2, II:5, III:6, and III:7 were analyzed by whole exome sequencing. Analysis of shared variants identified several hundred that were novel (not in ESP or 1,000 g databases) or rare (MAF <0.1%), predicted to be damaging by at least one in silico algorithm (SIFT, PolyPhen2, MutationTaster, and AlignGVD) and found in genes with a potential role in cardiac development or function. To determine which of the shared variants were most likely causative, they were prioritized using VAAST and pVAAST and then Phevor. This analysis found two variants of interest with higher scores than any other (laminin (β-2 chain (*LAMB2*) c.1750C>T: p.Arg584Cys, and myosin heavy chain 6 (*MYH6*) c.5653G>A, p.Glu1885Lys), with the *MYH6* variant having the highest rank (Fig.

3 and Table I). Because none of the variants reached genome wide significance threshold for pVAAST (2.54×10^{-6}), and Phevor is a non-parametric test, we had to consider many of the top ranked genes. This was done through additional genetic screening of un-sequenced family members (see below). An additional eight variants ranked by pVAAST or Phevor could not be eliminated as WPW candidates because of their reported function and/or cardiac expression (Table I). In addition, a variant in *KCNE2* (p.Ile57Thr), previously associated with Long QT syndrome [Abbott et al., 1999] and present in ClinVar as a disease-causing variant (http://www.ncbi.nlm.nih.gov/clinvar/RCV000006426/), was identified and considered a candidate because of this association, even though it was not ranked (Table I]. All available family members (both affected and unaffected) were screened to determine segregation of these variants. The *MYH6* variant was the only variant present in all affected individuals in the extended pedigree (including 1.2) and absent in all unaffected family members available for screening (Fig. 1). Of note, the *KCNE2* variant was not detected in two of the affected individuals and was present in four of the unaffected family members.

To further confirm the results of the VAAST analysis, results from the screens of family members who were not analyzed by WES were placed into a second pVAAST/Phevor analysis (Supplemental Fig. S1). This analysis confirmed that *MYH6* is by far the most likely causative gene among the top scoring candidates. When considered with the additional screened family members, the pVAAST *P*-value is very close to genome wide significance ($P = 9.77 \times 10^{-6}$).

In addition to having the highest score in Phevor, the *MYH6* variant is predicted to be likely deleterious/damaging by four commonly used in silico algorithms: Align GVD, SIFT, Polyphen2, and MutationTaster (Table I). This missense mutation (p.Glu1885Lys) alters an amino acid residing in the myosin tail domain and is perfectly conserved between species from zebrafish to humans (Fig. 4).

The distinct cohort of 47 unrelated WPW patients was screened for the mutations in *PRKAG2* and *MYH6*. Two novel synonymous variants were identified in *PRKAG2* (c. 231C>T: p.Phe77 and c.1485C>A. p.Thr495 (NM_016203.3)): neither are predicted to alter splicing. One novel non-synonymous variant was identified in *MYH6* (c.635C>T; p.Ala212Val), which is predicted to be benign/ not damaging by AlignGVD, SIFT, MutatioTaster, and Polyphen2. Therefore, neither *PRKAG2* nor *MYH6* are commonly mutated in our cohort of patients with WPW, confirming a high degree of genetic heterogeneity in this disease.

DISCUSSION

This study represents the first reported exome sequence analysis of a family with WPW. This family was initially screened for mutations in *PRKAG2*, the only gene robustly associated with WPW, and was negative for candidate mutations. We, therefore, performed WES on five family members (three affected, one unaffected, and an obligate carrier) in order to identify the disease-causing gene in this highly penetrant kindred. We employed recently designed prioritization strategies (VAAST/ pedigree VAAST) coupled with the ontology-based algorithm Phevor to generate a limited number of potential candidate alleles.

This strategy was previously successful in identifying disease-causing alleles in small, family-based WES analyses [Singleton et al., 2014]. Of the highly ranked alleles identified in our WPW family, only one segregated with the phenotype: *MYH6* c.5653G>A; p.Glu1885Lys. This variant affects a highly conserved glutamic acid in the myosin tail, is predicted to be pathogenic/deleterious by four separate in silico algorithms and is ranked as the strongest candidate variant by Phevor [Singleton et al., 2014]. This evidence supports the conclusion that the *MYH6* variant is the most likely causative mutation responsible for the WPW phenotype in this family, although there was variable/incomplete penetrance in this family. The *KCNE2* variant (I57T) is listed in ClinVar as a pathogenic variant. However, given the lack of segregation with phenotype in this WPW family, it is likely that this variant is not pathogenic or a modifier, at best.

These data expand the cardiac phenotypes associated with mutations in *MYH6*. The original association of *MYH6* mutations with cardiac disease was in a family with hypertrophic cardiomyopathy (HCM) [Niimura et al., 2002]. No members of the family studied here had signs of HCM: one patient (II.2) had mild septal hypertrophy but this was probably due to the elevated pressure within the left ventricle as a result of a subaortic membrane, rather than HCM. Subsequently, there have been other reports of mutations associated with HCM and dilated cardiomyopathy [Carniel et al., 2005]. In 2005, Ching et al. (2005) reported the identification of a mutation in *MYH6 in* a family with atrial septal defects (ASD): other *MYH6* mutations have also been linked to ASD [Granados-Riveron et al., 2010; Arrington et al., 2012].

In addition to the role of Myh6 in structural heart disease, variations in *MYH6* have previously been linked to cardiac arrhythmias and heart rate, but not pre-excitation. In a prior study to identify variants that modulate heart rate, PR interval and QRS duration in individuals of European descent, a large genome-wide association study (GWAS) identified *MYH6* as a locus linked to heart rate [Holm et al., 2010], and this was confirmed in a recent replication study [den Hoed et al., 2013]. In a GWAS study of Icelandic patients with sick sinus syndrome (SSS), Holm et al. (2011) identified an association with SNPs at chromosome 14q11. Whole genome sequencing analysis of four patients carrying one of the associated SNPs identified a missense variant in *MYH6* (c.2161C>T, p.Arg721Trp). Analysis of additional Icelandic patients and controls identified this variant in 2.1% of SSS patients but only 0.21% of controls. The authors calculated that the lifetime risk of being diagnosed with SSS is around 6% for non-carriers of *MYH6* c.2161C>T, but approximately 50% for carriers of the variant. In addition, there was a residual association, after exclusion of SSS cases, with other arrhythmias, including atrial fibrillation.

There is also an indirect link between *MYH6* and arrhythmias. The microRNA, miR-208a, is encoded within an intron of *MYH6*. MiR-208a Tg mice develop cardiac arrhythmias, have abnormal cardiac conduction, and altered expression of the cardiac transcription factors, including GATA4, and connexin 40 [Callis et al., 2009]. Further, the expression of this microRNA directly correlates with the expression of *MYH6* [Diniz et al., 2013]. Therefore, mutations that alter *MYH6* mRNA stability could impact the expression of MiR-208a, and consequently downstream target genes.

In summary, we utilized whole exome sequencing in a high-risk pedigree to identify a novel candidate locus for WPW, *MYH6*. The variant identified in this family (p.Glu1885Lys) is novel, predicted to be deleterious, and potentially alters the structure of a highly conserved functional domain. This coiled-coil myosin heavy chain tail region is responsible for interactions between myosin molecules and provides the structural backbone of the thick filament [Strehler et al., 1986]. The major limitations of this study are (i) that while WES provides data for the coding regions of most genes in the human genome there are still other regions that are not well covered (Supplemental Table SI). Therefore, disease causing variants in these regions would be missed, (ii) No functional data are provided to support the conclusion, that the *MYH6* variant is causative. However, we believe the combination of bioinformatics approaches and the known role for this protein in cardiac disease makes this a very strong candidate. We did not identify variants in *MYH6* or *PRKAG2* in other individuals from our cohort of 47 unrelated WPW patients, underscoring the high degree of genetic heterogeneity in this condition and suggesting that additional investigation in large WPW cohorts will be needed to replicate this finding. It is unclear why mutations in *MYH6* cause such pleiotropic effects on cardiac structure and function, ranging from atrial septal defects, to cardiomyopathies, to cardiac arrhythmias. From the locations of the variants associated with these phenotypes, genotype-phenotype correlations are not obvious (Supplemental Fig. S2) and will require more detailed modeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by funds from the American Heart Association (12GRNT11090001: Bowles, P.I. and 10FTF3920017: Jou, P.I), National Institutes of Health (1X01HL115006: Bowles and Leppert, P.I.), and the Primary Children's Foundation (Gruber, P.I.). Portions of this work were supported by NIGMS R01GM104390 and NHGRI R44HG006579 (Yandell, P.I.). Brett Kennedy was supported by a training award from the National Center for Advancing Translational Sciences, National Institutes of Health (1ULTR001067). DNA extractions were performed in the University of Utah Center for Clinical and Translational Science, which is funded by Public Health Services research grant no. M01-RR00064 from the National Center for Research Resources, the Children's Health Research Center at the University of Utah, and the Clinical Genetics Research Program at the University of Utah. Exome sequencing was performed by the Baylor-Hopkins Center for Mendelian Genomics, which is funded by National Human Genome Research Institute (NHGRI) grant U54HG006542 to Dr. Jim Lupski.

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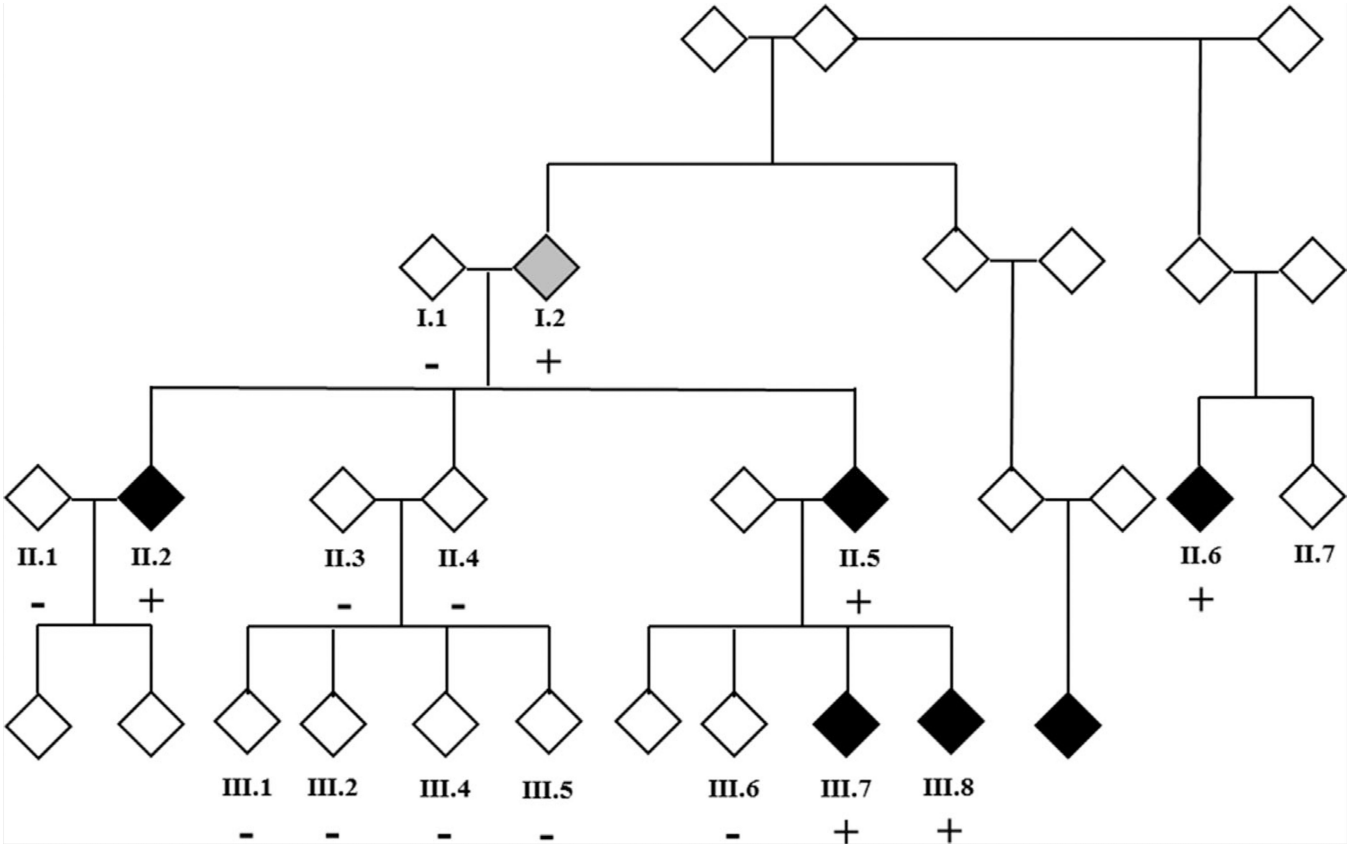

FIG. 1.

Family K32326 pedigree. The gender of family members is masked for confidentiality. Black symbols represent patients with WPW and a gray symbol represents the patient with a diagnosis of SVT (Patient 1:2): autosomal dominant inheritance, with incomplete penetrance, is the most likely genetic model. Only participants in the study for whom DNA is available for analysis are numbered. +: Positive for *MYH6* c.5653G>A, p.Glu1885Lys; −: Negative for *MYH6* c.5653G>A, p.Glu1885Lys.

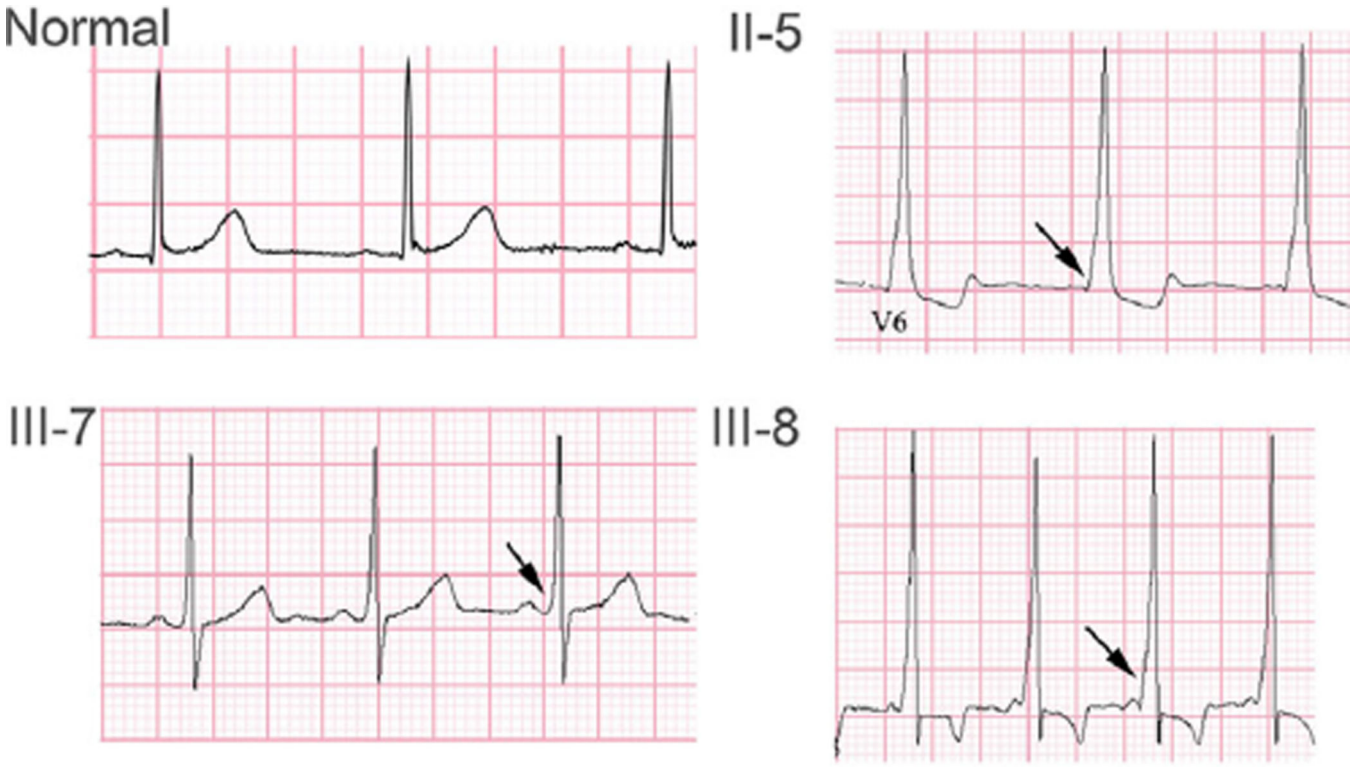

FIG. 2.

Representative ECGs from selected members of K32326. Top row: example of a normal resting ECG (left panel). Right panel, resting ECG from patient II-5 in sinus rhythm, showing classic short PR interval and ventricular pre-excitation or delta wave (arrow). Bottom row: ECG from patient III-7 shows short PR interval and subtle ventricular pre-excitation (left panel). ECG from patient III-8 reveals markedly short PR interval and prominent ventricular pre-excitation (right panel).

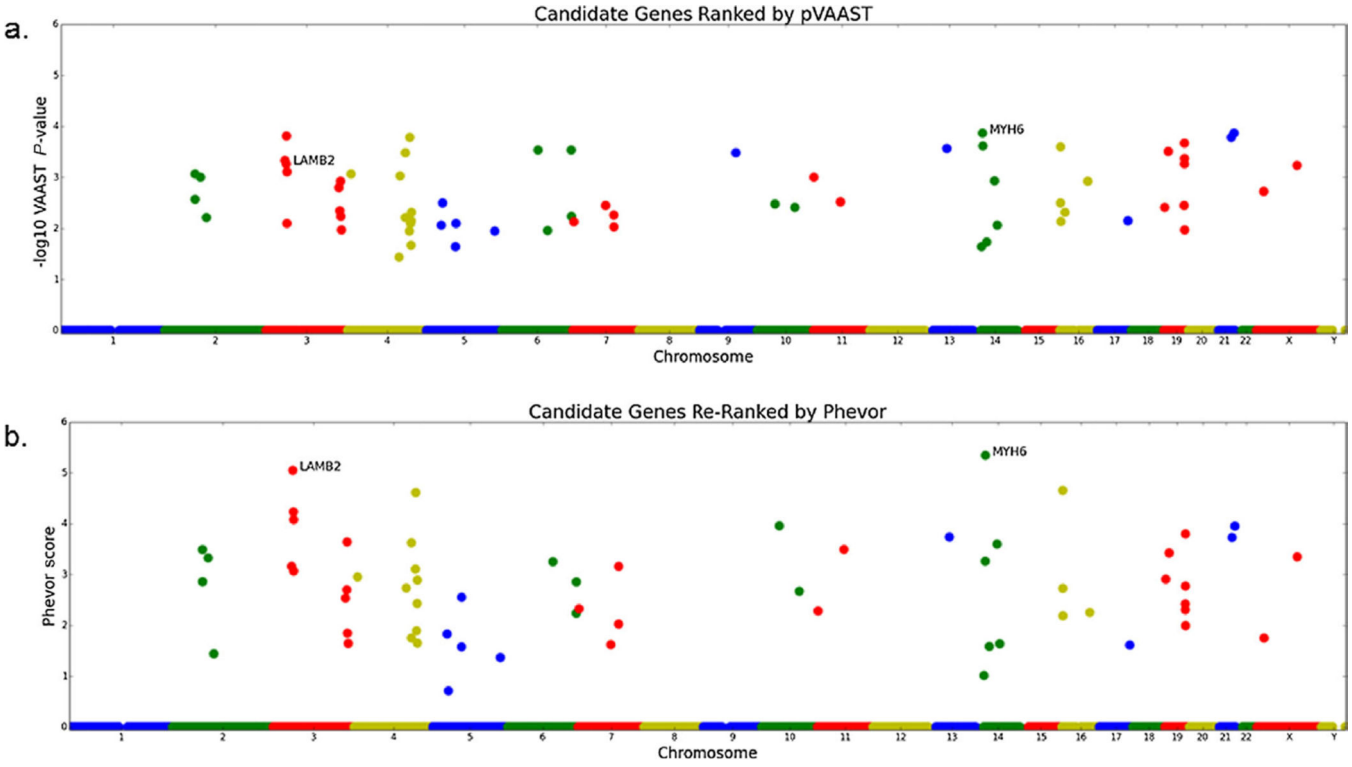

FIG. 3.

Manhattan plots of the pVAAST and Phevor results. A: Manhattan plot of pVAAST scores for all protein-coding genes in Human Genome release hg19; each dot is a single gene. B: Manhattan plot of Phevor scores obtained by using the pVAAST results in conjunction with the phenotype terms describing symptoms of Wolff–Parkinson–White. The *x*-axis shows the genomic location of each gene arranged by position along the chromosomes. The *y*-axis is the Phevor or pVAAST score. *MYH6* and *LAMB2* are highlighted in each plot as they are the highest scoring genes by the combination of analyses.

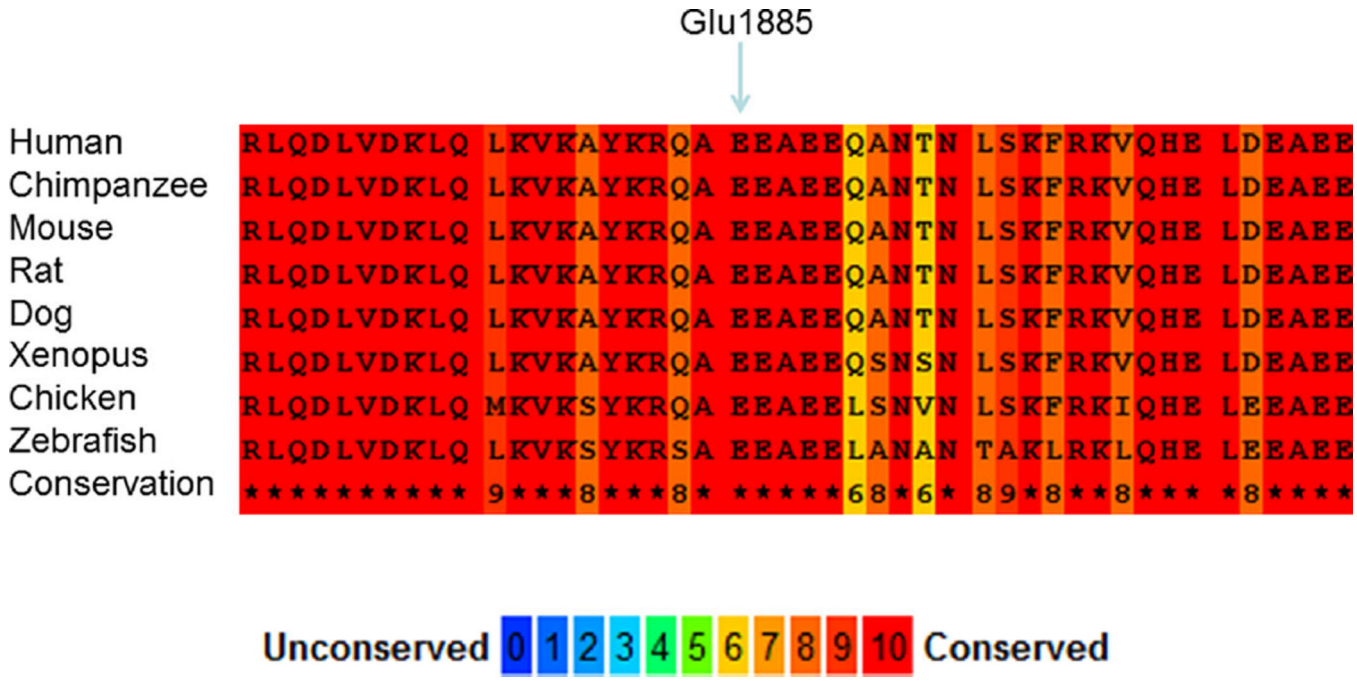

FIG. 4.

Conservation analysis of MYH6. Alignments of MYH6 across several species of the amino acids surrounding Glutamic acid 1885 generated using the PRALINE multiple sequence alignment tool (http://www.ibi.vu.nl/programs/pralinewww/). [Heringa, 1999] The scoring scheme is from 0 for the least conserved alignment position, up to 10 for the most conserved alignment position (represented by *) and the color assignments for each score are shown at the bottom. Note the complete conservation of this amino acid and the high degree of conservation across the domain.

TABLE I

Summary of Variants Ranked by pVAAST/Phevor Showing Pathogenicity Predictions of Four In Silico Algorithms

Gene	Phevor rank	pVAAST rank	Variant (transcript level)	Variant (protein level)	AlignGVD score	SIFT score	Polyphen2 score	MutationTaster score
MYH6	1	2	NM_002471.3: c.5653G>A	p. Glu1885Lys	C55	Deleterious (0.00)	Possibly damaging (0.716)	Disease causing (1.0)
LAMB2	2	17	NM_002292.3: c.1750C>T	p. Arg584Cys	C65	Deleterious (0.00)	Probably damaging (0.997)	Disease causing (1.0)
CCDC154	3	8	NM_001143980.1: c.959T>A	p. Leu320Gln	C65	Deleterious (0.00)	Probably damaging (0.999)	Polymorphism (0.940)
IFRD2	5	3	NM_006764.4: c.1175G>A	p. Arg392His	C0	Tolerated (0.10)	Probably damaging (0.945)	Disease causing (0.983)
DIP2A	8	1	NM_015151.3: c.3319C>T	p. Arg1107Trp	C0	Deleterious (0.01)	Probably damaging (0.948)	Disease causing (0.995)
SIGLEC11	9	18	NM_052884.2: c.342C>G	p. Cys114Trp	C15	Deleterious (0.00)	Probably damaging (1.000)	Disease causing (1.0)
LTF	22	16	NM_002343.3: c.293C>T	p.Ala98Val	C0	Tolerated (0.07)	Possibly damaging (0.689)	Polymorphism (0.554)
FUZ	32	6	NM_025129.4: c.272C>T	p.Ser91Phe	C0	Deleterious (0.02)	Probably damaging (0.946)	Disease causing (0.998)
INTU	33	23	NM_015693.3: c.2512T>C: rs144025772	p. Cys838Arg	C0	Deleterious (0.01)	Probably damaging (0.974)	Disease causing (1.000)
MYH14	42	15	NM_001145809.1: c.1919G>A: rs199696801	p. Arg640Gln	C0	Deleterious (0.04)	Benign (0.055)	Polymorphism (0.934)
KCNE2	-	-	NM_172201.1: c.170T>C: rs74315448	p.lle57Thr	C65	Deleterious (0.00)	Probably damaging (0.918)	Disease causing (0.999)

Note: AlignGVD scores variants on a scale C0, C15, C25, C35, C45, C55, C65, with CG5 representing the highest risk allele. The *KCNE2* variant was not ranked by Phevor but was considered a candidate variant because of its association with Long OT syndrome [Abbott et al., 1999] and is present in ClinVar as a disease-causing variant (http://www.ncbi.nlm.nih.gov/clinvar/RCV000006426/).