Functional assessment of potential splice site variants in arrhythmogenic right ventricular dysplasia/cardiomyopathy

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BACKGROUND Interpretation of genetic screening results in arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) often is difficult. Pathogenicity of variants with uncertain clinical significance may be predicted by software algorithms. However, functional assessment can unambiguously demonstrate the effect of such variants.

OBJECTIVE The purpose of this study was to perform functional analysis of potential splice site variants in ARVD/C patients.

METHODS Nine variants in desmosomal (*PKP2*, *JUP*, *DSG2*, *DSC2*) genes with potential RNA splicing effect were analyzed. The variants were found in patients who fulfilled 2010 ARVD/C Task Force Criteria (n = 7) or had suspected ARVD/C (n = 2). Total RNA was isolated from fresh blood samples and subjected to reverse transcriptase polymerase chain reaction.

RESULTS An effect on splicing was predicted by software algorithms for all variants. Of the 9 variants, 5 were intronic and 4 exonic. RNA analysis showed a functional effect on mRNA splicing by exon skipping, generation of new splice sites, or activation of cryptic sites in 6 variants. All 5 intronic variants tested severely impaired splicing. Only 1 of 4 exonic potential splice site variants was shown to have a deleterious effect on splicing. The remaining 3 exonic variants had no detectable effect on splicing, and heterozygous presence in mRNA confirmed biallelic expression.

CONCLUSION Six variants of uncertain clinical significance in the *PKP2*, *JUP*, and *DSG2* genes showed a deleterious effect on mRNA splicing, indicating these are ARVD/C–related pathogenic splice site mutations. These results highlight the importance of functional assessment of potential splice site variants to improve patient care and facilitate cascade screening.

KEYWORDS Arrhythmogenic right ventricular dysplasia/cardiomyopathy; Genetics; Splice site mutations; Ventricular arrhythmias; RNA analysis; Desmosomes

ABBREVIATIONS **ARVD/C** = arrhythmogenic right ventricular dysplasia/cardiomyopathy; **DSC2** = desmocollin-2; **DSG2** = desmoglein-2; **DSP** = desmoplakin; **JUP** = plakoglobin; **LBBB** = left bundle branch block; **LV** = left ventricle; **MAF** = minor allele frequency; **PKP2** = plakophilin-2; **PLN** = phospholamban; **RT-PCR** = reverse transcriptase polymerase chain reaction; **RV** = right ventricle; **TFC** = Task Force Criteria for ARVD/C diagnosis; **TMEM43** = transmembrane protein-43; **VT** = ventricular tachycardia; **VUS** = variants of uncertain clinical significance

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Introduction

Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) is a hereditary cardiomyopathy characterized by

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ventricular arrhythmias, right ventricular (RV) and possibly left ventricular (LV) dysfunction, and fibrofatty replacement of cardiomyocytes.[1–3] The genetic substrate has been demonstrated in 5 desmosomal genes and, in a minority, in nondesmosomal genes.[4–8] A pathogenic ARVD/C–related mutation can be identified in 30%–70% of index patients.[9–11] A large web-based dataset of genes and mutations associated with ARVD/C has been created (www.arvcdatabase.info).[12] The identification of a pathogenic mutation in index patients with ARVD/C facilitates cascade screening of family members and subsequent identification of subjects with a disease predisposition. However, not infrequently genetic screening also results in the identification of gene variants of uncertain clinical significance (VUS).[13] The uncertainty of the finding of VUS undermines risk management in ARVD/C and poses a burden on patients and physicians.

The possible pathogenicity of VUS is routinely estimated by *in silico* prediction algorithms. However, the clinical relevance of a VUS may be determined by experimental validation *in vitro*, specifically with potential splice site variants. Not only variants affecting the invariant splice donor and splice acceptor splice sites but also more distant intronic and even nonsense, missense, or translationally silent exonic variants can impair gene activity by inducing the splicing machinery to skip the variant-bearing exons and/or activate cryptic splice sites.[14–16] Variants of which the spliceogenic potential is not immediately recognized by prediction algorithms may be incorrectly predicted as innocuous, as demonstrated previously.[16,17] To date in ARVD/C, the spliceogenic character of only a few splice site mutations was experimentally confirmed.[18,19]

In this study, we analyzed 9 distinct ARVD/C VUS for functional impact on splicing by bioinformatic prediction tools and experimental analysis of mRNA. We aimed to confirm or discard pathogenicity of these 9 potential splice site variants for patient management purposes and to improve knowledge on ARVD/C splice site mutations.

Methods

Study population

Nine variants with spliceogenic potential, defined as an anticipated pathogenic effect on splicing according to *in silico* algorithms that were identified by routine genetic screening as part of the diagnostic evaluation in ARVD/C, were assessed. The potential splice site variants were identified in 9 index patients, 7 with proven (definite) ARVD/C and 2 index patients with suspected ARVD/C. Proven ARVD/C was defined as fulfillment of 2010 Task Force Criteria (TFC) for diagnosis. ARVD/C diagnosis is made by the fulfillment of 2 major TFC, 1 major and 2 minor TFC, or 4 minor TFC in different categories: global and/or regional RV dysfunction and structural alterations, tissue characterization, depolarization abnormalities, repolarization abnormalities, ventricular arrhythmias, and family history/genetics.[20] Suspected ARVD/C was defined as fulfillment of 1 major or ≥ 1 minor TFC. One patient with suspected ARVD/C and a potential desmoglein-2 spliceogenic variant was subsequently shown to have the pathogenic phospholamban (*PLN*) founder mutation c.40_42delAGA (p.Arg14del). None of the other patients had additional pathogenic mutations. All patients consented to clinical and genetic evaluation and in particular to DNA and RNA analysis. The study conformed to the Guiding Principles of the Declaration of Helsinki and was approved by the Institutional Review Board at our institution.

Molecular genetic analyses

DNA was isolated from peripheral blood lymphocytes according to standard protocols. The coding regions of the desmosomal genes plakophilin-2 (*PKP2*), desmoplakin (*DSP*), plakoglobin (*JUP*), desmoglein-2 (*DSG2*), desmocollin-2 (*DSC2*), and nondesmosomal genes transmembrane protein 43 (*TMEM43*) and phospholamban (*PLN*) were analyzed using direct Sanger sequence analysis performed with a BigDye Terminator DNA sequencing kit (version 2.0) on a 3730 automated sequencer (Applied Biosystems, Foster City, CA). All sequences were analyzed using established sequence analysis software.

Total RNA was isolated from fresh blood samples from the ARVD/C index patients using the PAXgene kit (QIAGEN Benelux, Venlo, The Netherlands) and subjected to random hexamer primed reverse transcriptase polymerase chain reaction (RT-PCR). The obtained cDNA products were amplified by PCR with exonic primers specific for the fragment of interest. All cDNA fragments were separated according to size using 2% agarose gel electrophoresis along with a 100-bp DNA ladder (O'GeneRuler; Fermentas, Burlington, Ontario, Canada). Both normal and aberrant fragments were subjected to direct sequencing. Primers and PCR details are available upon request.

Prediction of pathogenicity

The pathogenic potential of the splice site variants was assessed using *in silico* prediction analyses of (1) the maximum entropy model (MaxEntScan),[21] (2) the Human Splicing Finder (HSF),[22] (3) NNSPLICE,[23] (4) GeneSplicer,[24] and (5) SpliceSiteFinder-like.[25] Variant minor allele frequency (MAF) was assessed in large control databases, including Dutch control datasets (NHLBI 6500 exome dataset [EVS], http://evs.gs.washington.edu/EVS/, and Genome of the Netherlands dataset [GoNL], http://www.nlgenome.nl/). Variant prevalence was also reviewed in the Dutch ARVD/C cohort, a clinical dataset based on national collaboration of all university medical centers in The Netherlands.

Results

Molecular genetic analyses

Routine diagnostic screening of desmosomal genes *PKP2*, *DSP*, *JUP*, *DSG2*, and *DSC2* and nondesmosomal genes *TMEM43* and *PLN* identified 9 distinct variants with spliceogenic potential (Table 1). In all variants at least 1 *in*

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