

Impact of RNA testing on cardiac variant interpretation and patient management



Lior Jankelson, MD, PhD,* Heather Zimmermann, PhD,[†] Steven Fowler, MD,*¹
Linda Borneman, NP,* Blair Conner, MS,[†] Sitao Wu, PhD,[†] Rachid Karam, MD, PhD,[†]
Larry Chinitz, MD,* Marina Cerrone, MD*

From the *Leon H. Charney Division of Cardiology, NYU School of Medicine, New York, New York, and
[†]Ambry Genetics, Aliso Viejo, California.

Introduction

The identification of the substrate of idiopathic ventricular fibrillation (VF) in the absence of structural heart disease or electrocardiographic changes remains a challenge for patient management and family counseling. The availability of expanded genetic testing panels and clinical exome screening has not led to a significant increase in the identification of genetic causes of these events, as shown by recent reports.^{1,2} Indeed, the majority of studies detected only a small number of genetic variants^{1,2} that could explain a sudden cardiac arrest event. Additionally, most variants still remain classified as “of unknown significance” and do not provide information useful for mechanistic understanding and cascade screening. Variants of unknown significance (VUS) of particular complex adjudication are those located at, and possibly altering, RNA splicing sites. RNA testing is thus critical in characterizing splicing alterations and could provide more precise insights compared to in silico prediction algorithms alone. We present here a case of idiopathic VF where RNA studies on the patient’s blood cells helped revise the adjudication of a VUS, providing valuable insights and implications for cascade screening.

Case report

A 38-year-old white man experienced out-of-hospital cardiac arrest while playing basketball recreationally. He collapsed suddenly while running, with loss of consciousness and limb jerking. The presenting rhythm was VF and successful defibrillation to sinus rhythm was provided by emergency medical services. The patient was transferred to a critical

care unit where he underwent hypothermia induction. A complete diagnostic work-up including electrocardiogram (ECG) (Figure 1A), echocardiogram, and cardiac magnetic resonance imaging revealed normal cardiac structure and function. Cardiac catheterization confirmed absence of significant coronary artery disease/thrombosis. He declined procainamide challenge and his ECG was not suspicious for alterations consistent with Brugada syndrome. His medical history was positive for a C282Y homozygous mutation in the *HFE* gene associated with hereditary hemochromatosis. His iron load was controlled through periodic blood donations. At the time of hospitalization, cardiac magnetic resonance imaging and abdominal ultrasounds did not reveal evidence of iron deposition, although tissue biopsies had not been performed. His father and sister were also affected by hereditary hemochromatosis, clinically controlled through blood donations.

A single-chamber implantable cardioverter-defibrillator was implanted for secondary prevention. A stress test performed 2 months after the event was negative for ischemic changes, arrhythmias, and/or QT prolongation. During the 18 months of follow-up, rare runs of asymptomatic atrial tachycardia (longest 2 minutes) in the absence of ventricular arrhythmias were noted. Repeated ECGs showed consistently normal depolarization and repolarization (Figure 1B). Family history was negative for sudden death, arrhythmia, or seizure disorder.

Genetic test results

Genetic testing was performed via a commercially available panel (comprehensive arrhythmia panel including 39 genes associated with inherited arrhythmias, Ambry Genetics, Aliso Viejo, CA). Three variants, all initially classified as VUS, were detected in the *AKAP9*, *CACNA1C*, and *LMNA* genes, coding for the A-kinase anchor protein 9, the α -subunit of the cardiac calcium channel $Ca_v1.2$, and lamin A/C, respectively. The *AKAP9* variant (c.1259A>G, p.Q420R) had not been previously reported in the literature. It is present at a very low minor allele frequency in the gnomAD database³ (0.0055%), and in silico analyses predicted it to result

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Dr Zimmermann, Mr Conner, Dr Wu, and Dr Karam are full-time employees of Ambry Genetics. The authors have no additional disclosures.
¹Dr Fowler is currently affiliated with the Community Hospital of Monterey Peninsula, Monterey, California. **Address reprint requests and correspondence:** Dr Marina Cerrone, Leon H. Charney Division of Cardiology, NYU School of Medicine, Science Building, 435 E 30th St 723H-3, New York, NY 10016. E-mail address: marina.cerrone@nyumc.org.

KEY TEACHING POINTS

- Functional assessment of splice site variants in vivo is essential to adjudicate potential involvement as a substrate for idiopathic cardiac arrest and is superior to computational prediction tools.
- Incidental genetic findings in the setting of idiopathic ventricular fibrillation should be interpreted with caution in the absence of functional data and clear clinical phenotype.
- Cascade screening in the presence of variants of unknown significance without a strong functional characterization should not be performed.

in a benign physicochemical change. Importantly, *AKAP9* mutations have been linked to the rare LQT11, which is responsible for <1% of long QT syndrome cases.⁴ The patient's QT interval was normal, ranging from 390 to 430 ms in different recordings, including during exercise.

The *CACNA1C* missense change (c.6272A>G, p.N2091S) is present in the gnomAD population database with a relatively high minor allele frequency of 0.048%. *CACNA1C* variants have been linked to multiple arrhythmic phenotypes: Brugada syndrome, short QT syndrome, Timothy syndrome, and isolated long QT syndrome forms.⁴ In the literature, it has been reported as an incidental finding in 1 patient with history of syncope and sudden death, without clinical features consistent with either Timothy or Brugada syndrome.⁵ Published in vitro characterization in HEK293 cells resulted in increased calcium current, which could be consistent with a long QT phenotype. However, this patient did not show QT prolongation at multiple recordings. Phylogenetic conservation across species is weak for the arginine reference amino acid and in silico prediction for the variant provided contrasting interpretations.

Both variants remained inconclusive and we thought they had a limited probability of being causative of the patient's clinical presentation.

The third variant, *LMNA* c.480C>T, p.G160G, was described as possibly disrupting RNA splicing. Its frequency in gnomAD is rare (0.00086% in white populations), and it has not been previously reported in any patient series. Multiple in silico splice site prediction tools (BDGP, ESEFinder, Human Splicing Finder, MaxEnt) postulated that this synonymous variant creates a strong alternative splice donor site that may compete with the native site. This alternative donor site would lead to the deletion of the last 35 nucleotides of exon 2, resulting in a frameshift with predicted p.G160Afs*2 that would likely be subject to nonsense-mediated decay. In support of the in silico predictions, this variant was recently studied through an in vitro minigene assay in HEK293 cells and was reported to have a deleterious

impact on splicing, with only 27.4% of transcripts undergoing a normal splice event in the assay.⁶

Functional assessment of the *LMNA* splice variant

LMNA variants have been linked to cases of cardiac arrest even in the absence of additional manifestations of *LMNA*-associated diseases. We thus investigated whether the p.G160G splice change indeed had significant impact on splicing in vivo, as suggested by in silico and in vitro assays. Informed consent was obtained and the study was approved by the Western Institutional Review Board for Ambry Genetics and the NYU Institutional Review Board (study 09-0297). Peripheral blood from the patient and normal healthy controls was collected in PAX gene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) and stored according to the manufacturer's recommendations. Total RNA was isolated using the PAX gene Blood RNA kit. Patient and control cDNA was then generated from mRNA with the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA). Commercially available human heart cDNA pooled from 3 white men was used as a tissue-specific control (Clontech, Mountain View, CA). Polymerase chain reaction (PCR) was performed with HotstarTaq (Qiagen, Hilden, Germany) using a forward primer located in exon 1 and a reverse primer spanning the exon 4/exon 5 splice junction (5'-CAAGACCCTTGACTCAGTAGC-3' and 5'-TGGCATTGTCCAGCTTGG-3'). PCR products were visualized with digital electrophoresis using D1000 ScreenTape and Reagents on TapeStation 2200 (Agilent, Santa Clara, CA).

As shown in Figure 2A, no aberrant splicing was detected by digital electrophoresis of the reverse transcriptase (RT)-PCR products in either the patient or control samples. The RT-PCR products were subsequently cloned using the CloneSeq protocol and analyzed following the bioinformatic platform described by Farber-Katz and colleagues.⁷ CloneSeq is a highly sensitive and highly specific assay that offers a high-resolution picture of splice events occurring at this locus, allowing for both quantitative and qualitative analysis of the cloned RT-PCR products.⁷

Alternative splicing was negligible (<1% of reads) in all samples as illustrated by the Sashimi plots in Figure 2B, which provide a visualization of the aligned massively parallel sequencing reads. In addition, the mutated (T) allele was present in 47% (1706/3619) of wild-type reads in the patient sample, indicating there was no allele imbalance. Hence, taken together, the data point to a lack of significant impact on splicing by the p.G160G *LMNA* variant in vivo.

Discussion

Here we describe a case of idiopathic VF with an incidental finding of an *LMNA* variant predicted to disrupt splicing and protein integrity by in silico algorithms. Published in vitro characterization of this variant with a minigene assay

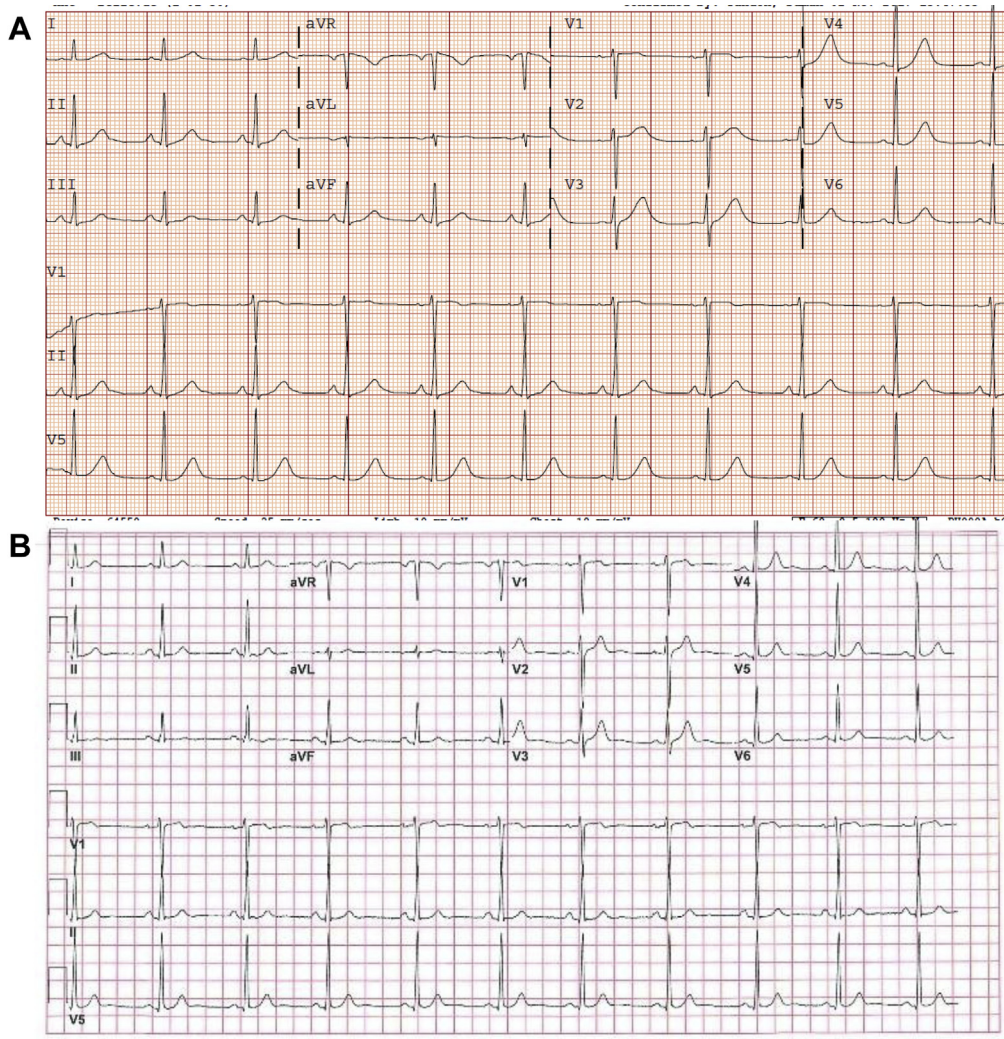


Figure 1 Electrocardiograms recorded **A:** at admission and **B:** at 6 months follow-up, confirming normal depolarization and repolarization.

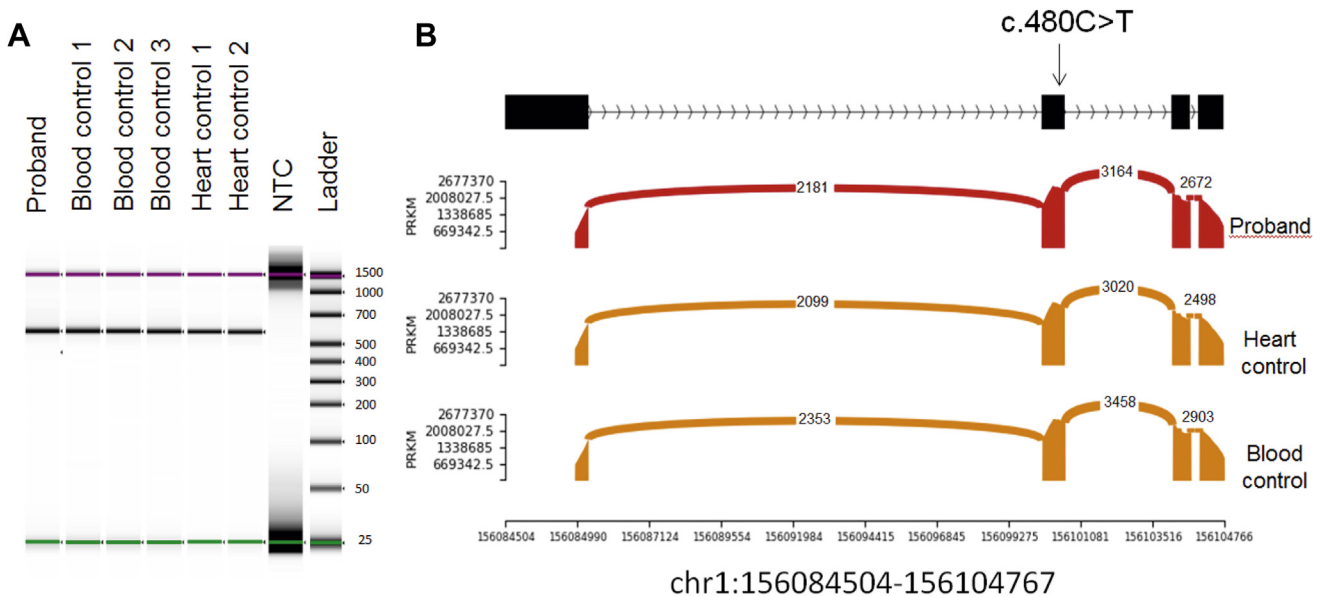


Figure 2 **A:** Digital electrophoresis analysis of reverse transcriptase polymerase chain reaction (RT-PCR) performed on blood from the proband and 3 healthy controls does not detect abnormal splicing. RT-PCR on control heart samples gave similar results. **B:** Sashimi plots of aligned massively parallel sequencing reads from CloneSeq for splicing events detected in >1% of reads. The number of reads exhibiting a given splice event is shown. NTC = no template control.

suggested it had a deleterious impact on splicing.⁶ However, functional characterization using the RNA derived from the patient's peripheral blood cells failed to confirm aberrant splicing *in vivo*, thus demoting the possible role of the variant as causative of the clinical phenotype.

LMNA variants are associated with familial dilated cardiomyopathy, progressive conduction disease, ventricular arrhythmias, and increased risk of sudden death.^{4,8} The phenotype penetrance is age-dependent, and there are descriptions of cases in which the initial clinical manifestation has been VF without manifest cardiomyopathy or conduction delay, especially in male individuals with nonmissense mutations.^{8–10} Variants affecting splicing tend to be considered as potentially disruptive of protein function. Indeed, the ClinVar database currently lists 30 *LMNA* splicing variants and among those with an interpretation, 1 of 26 are classified as VUS while the remainders are considered pathogenic/likely pathogenic.

Hence, the *LMNA* c.480 C>T was the one judged most likely to be causative of the phenotype in the patient.

The substrate in the majority of cases of isolated VF occurring in young individuals without a clear clinical phenotype remains undiagnosed. The yield of genetic test for inherited channelopathies and/or cardiomyopathies, in the absence of clinical diagnosis, even using whole exome screening, is extremely low and the majority of variants detected falls within the VUS category.^{1,2}

Although cardiac arrest survivors are usually protected from the recurrence of fatal events by implantable cardioverter-defibrillators, the quest for a genetic substrate has important implications for family screening and identification of individuals at risk. A VUS should be considered a not actionable variant in terms of clinical management and cascade screening.¹¹ However, it is not uncommon for cascade screening to be performed following detection of some VUS even if discouraged by the current recommendations of the American College of Medical Genetics.

Among VUS of complex interpretation, the ones affecting splice sites remain very challenging. This is in part related to the difficulties of characterizing them *in vitro*, which relies on technology available in only a few select research labs and is both time- and resource-intensive, while high-throughput assays are scarce. Furthermore, standard RT-PCR followed by Sanger sequencing is at best semiquantitative and may not detect minor splice isoforms, while *in vitro* minigene assays do not always accurately reflect splicing *in vivo* owing to limitations of the method. Minigene assays do not faithfully duplicate the endogenous genomic and cellular environments, which may contain *cis*-acting regulatory elements, splicing factors, or other determinants that influence splicing *in vivo*. Use of the CloneSeq protocol⁷ allowed us to obtain a high-resolution picture of splicing at the relevant exon/intron junction *in vivo*. We were able to qualitatively and quantitatively determine that transcripts derived from the variant allele were not subject to aberrant splicing.

Peripheral blood cells are an excellent and easily accessible source of RNA, if the gene of interest for a determinate tissue is sufficiently expressed. Though this represents a major limitation for several genes of cardiac interest, *LMNA* expression in bone marrow and lymph node is satisfactory (<https://www.proteinatlas.org/ENSG00000160789-LMNA/tissue>). No cardiac-specific isoforms for *LMNA* are known and the 2 major isoforms (lamin A and C) are expressed in almost all differentiated cells.¹² In addition, we observed no differences in *LMNA* splicing between control and heart samples in our assay (Figure 2), confirming that blood is a suitable RNA source for assessing *LMNA* splicing alterations. The CloneSeq program has been successfully applied to the study of splice variants in actionable genes of interest for increased cancer risk.⁷ Here we show that this approach is feasible also for genes linked to heritable cardiac arrhythmias and could be helpful in adjudicating VUS.

It is not yet clear why our patient experienced sudden cardiac arrest. There are cases of cardiac arrest, syncope, and life-threatening ventricular arrhythmias that have been linked to hereditary hemochromatosis.^{13,14} The mutation C282Y carried by the patient is the most commonly detected pathogenic alteration related to hemochromatosis in white populations and could have variable phenotypic expression and penetrance.^{13,15} There are preliminary reports suggesting that this mutation could be associated with increased arrhythmic burden even in the absence of macroscopic iron deposition in the myocardium.¹⁵ Although the ultimate diagnosis in this patient remains “idiopathic” VF with unknown causes, the exclusion of a role for the *LMNA* variant carries important implications in terms of cascade screening and calls for caution in the assessment of genetic variants in the absence of careful functional characterization.

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