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ORIGINAL ARTICLE

MicroRNAs in cardiac arrhythmia: DNA sequence variation of MiR-1 and MiR-133A in long QT syndrome

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Abstract

Long QT syndrome (LQTS) is a genetic cardiac condition associated with prolonged ventricular repolarization, primarily a result of perturbations in cardiac ion channels, which predisposes individuals to life-threatening arrhythmias. Using DNA screening and sequencing methods, over 700 different LQTS-causing mutations have been identified in 13 genes worldwide. Despite this, the genetic cause of 30–50% of LQTS is presently unknown. MicroRNAs (miRNAs) are small (~22 nucleotides) noncoding RNAs which post-transcriptionally regulate gene expression by binding complementary sequences within messenger RNAs (mRNAs). The human genome encodes over 1800 miRNAs, which target about 60% of human genes. Consequently, miRNAs are likely to regulate many complex processes in the body, indeed aberrant expression of various miRNA species has been implicated in numerous disease states, including cardiovascular diseases. MiR-1 and MiR-133A are the most abundant miRNAs in the heart and have both been reported to regulate cardiac ion channels. We hypothesized that, as a consequence of their role in regulating cardiac ion channels, genetic variation in the genes which encode MiR-1 and MiR-133A might explain some cases of LQTS. Four miRNA genes (*miR-1-1*, *miR-1-2*, *miR-133a-1* and *miR-133a-2*), which encode MiR-1 and MiR-133A, were sequenced in 125 LQTS probands. No genetic variants were identified in *miR-1-1* or *miR-133a-1*; but in miR-1-2 we identified a single substitution (*n.100A> G*) and in *miR-133a-2* we identified two substitutions (*n.−19G> A* and *n.98C> T*). None of the variants affect the mature miRNA products. Our findings indicate that sequence variants of *miR-1-1*, *miR-1-2*, *miR-133a-1* and *miR-133a-2* are not a cause of LQTS in this cohort.

Key Words: *DNA mutational analysis, single nucleotide polymorphism, gene expression regulation, long QT syndrome*

Background

Long QT syndrome (LQTS) is a genetic condition characterized by prolongation of the QT interval, syncopal attacks, T-wave abnormalities, ventricular tachycardia of the torsades de pointes (TdP) type and an increased risk of sudden death [1].

The population prevalence of LQTS is estimated to be between 1:2000 and 1:5000 [1,2] and the disease phenotype is associated with highly variable expressivity [3] and incomplete penetrance [4]. Presently, using genetic screening and DNA sequencing techniques, over 700 LQTS-causing mutations have been identified in 13 genes [5,6]. These genes are involved in the correct execution of the cardiac action potential. Genetic screening of the five most frequently affected genes (*KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1* and *KCNE2*) results in the identification of a disease-causing mutation in 50–70% of symptomatic LQTS cases [7]; the additional eight genes, which complete the list of LQTS-causative genes known presently, each account for very few LQTS cases [5,6]. A number of LQTS cases, with as-yet-unknown genetic aetiology, might be attributable to mutations in genes

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which regulate the expression of these LQTS-causing genes.

Initially characterized by Lee et al. [8] in 1993, microRNAs (miRNA) are first transcribed as long pri-miRNA transcripts containing a stem loop secondary structure, which, when excised by splicing or cleaved by the nuclear RNA cleaving enzyme, Drosher, is called pre-miRNA. Removal of the terminal loop from the pre-miRNA by the cytoplasmic RNA cleaving enzyme, Dicer, produces the small (~ 22 nucleotides) mature miRNA duplex [9]. Usually, one strand is preferentially selected for entry into the RNA-induced silencing complex (RISC), while the other strand, known as the miRNA* strand, is degraded. RISC is a multi-protein complex which uses the single stranded miRNA as a template for recognizing complementary messenger RNAs (mRNAs). The targeted transcripts are regulated through the binding of these complementary sequences and subsequent repression of gene expression through the regulation of mRNA translation or degradation [8,10–12]. The miRNAs of animals achieve this regulation through an imperfect association with mRNA target regions [13–15]. Furthermore, this incomplete complementarity with targets provides an opportunity for animal miRNA's to bind multiple different mRNA targets; similarly a given target might bind multiple miRNAs [16,17].

The human genome may encode over 1800 miRNAs [18,19], which target about 60% of human genes [13,20]. Consequently, miRNAs are likely to be involved in most biological processes [14,21]. Indeed, miRNAs have been reported to regulate many complex processes in the body and aberrant expression of various miRNA species has been implicated in numerous disease states including cancer [22], diabetes [23,24], systemic lupus erythematosus [25] and cardiovascular disease, e.g. heart failure, hypertrophy, conduction disturbances and arrhythmogenesis [26–35].

MiR-1 and MiR-133A are muscle-specific miRNAs, expressed predominantly in the heart and skeletal muscle [36]; they are the most abundant miRNAs expressed in the heart [30,37]. The bicistronic miRNA clusters encoding *miR-133a-1*/*miR-1-2* (located on chromosome 18) and *miR133a-2*/*miR-1-1* (located on chromosome 20) are regulated cooperatively by the myocyte enhancer factor-2 (MEF2) transcription factor and the serum response factor (SRF) which are essential regulators of muscle development [38,39]. The sequences of the mature *miR-1-1* and *miR1-2* are identical (MiR-1), as are those of *miR-133a-1* and *miR-133a-2* (MiR-133A). Targets of MiR-1 and MiR-133A include those mRNA transcripts encoding proteins involved in cardiovascular development [29], hypertrophy [26,27] and ion channel function [30]. Ion channels, such as *RYR2*, *KCNQ1* and *KCND2*, have been shown to be regulated, at least in part, by MiR-1 or MiR-133A [34,35,40].

MiR-1 was shown to play a role in cardiac morphogenesis and conduction in a genetic knockout mouse model [35]. Mice lacking miR-1-2 show an approximate 50% lethality between late embryogenesis and shortly after birth, as a consequence of ventricular septal defects. Postnatal survivors displayed a range of cardiac phenotypes, however while most displayed no structural dysfunction many suffered sudden death. Electrocardiography of mutant mice showed that many mutant carriers displayed prolonged ventricular depolarisation [35]. Mice lacking either MiR-133A gene did not exhibit cardiac defects nor was their life span reduced compared to control animals [41]. However, increased levels of MiR-133A prolong the action potential in isolated ventricular myocytes and cause QT prolongation in mice [31]. Furthermore, using a guinea pig model, Shan et al. could show that increasing MiR-1 and MiR-133A induced QT prolongation, while antisense silencing of MiR-1 and MiR-133A abolished Arsenic trioxyde-induced QT prolongation [42]. MiR-133A was shown to bind to the 3′UTR of KCNQ1 mRNA which encodes Kv7.1 [40]. Kv7.1 is the pore-forming subunit of the voltage-gated potassium channel which is responsible for the I_{Ks} cardiac potassium current. This current is essential for the repolarisation phase of the cardiac action potential, and mutations in *KCNQ1* are known to cause a loss-of-function phenotype, which manifests clinically as LQTS [5].

We hypothesized that genetic variation in MiR-1 or MiR-133A might explain some cases of LQTS as the phenotype is a result of loss-of-function and gain-of-function perturbations in cardiac ion channels.

Methods

Patient samples

Unrelated probands, referred from specialist cardiology centres in Denmark and the UK to Statens Serum Institut for genetic investigation of LQTS ($n = 125$; 70% female), were included in this retrospective study. These probands are primarily of Northern European descent (96%) and consequently, data from various European genetics screening panels (retrieved from NCBI dbSNP) were used as *in silico* control of allele frequencies.

The probands had been screened for mutations in *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1* and *KCNE2* by capillary-electrophoresis single strand conformation polymorphism (CE-SSCP) followed by DNA sequencing of aberrant conformers as described elsewhere [43–45]. Screening for mutations in *CAV3* was previously performed by direct sequencing [46]. Forty-eight (38%) had been found to carry a probably damaging variant in any one of these genes [47], these constitute the mutation carrier sub-group.

Those probands in whom no mutation was identified constitute the non-mutation carrier sub-group.

Ethics statement

This study was performed in accordance with the Helskini Declaration of 1975, as revised in 1983. All patients gave informed consent.

Genetic screening

DNA extraction. Genomic DNA (gDNA) was extracted from frozen EDTA-blood using a Qiagen kit (Qiagen, QmbH, Hilden, Germany).

Polymerase chain reaction. Four miRNA genes (*mir-1-1*, *mir-1-2*, *mir-133a-1* & *mir-133a-2*) were PCR amplified from 125 gDNA samples (Table I). Primers were designed using NCBI/Primer-BLAST program [48] (Table I); all primers were modified with M13 tails (F: TGTAAAACGACGGCCAGT; R: CAGGAAACAGCTATGACC).

A 25 μL PCR mix incorporated 1 U of TEMPase Hot Start DNA Polymerase (Ampliqon ApS), 1 ×Tempase buffer I (Ampliqon ApS), 2 mM dNTPs, 0.4 um of the forward and reverse primers, and 2.5 ng gDNA template.

PCR amplifications were performed in GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA, USA) using the following amplification protocol: initial heat activation step at 95°C for 15 min, 38 cycles of 30 s at denaturing temperature 94°C, 45 s at annealing temperature 58°C, and 40 s at extension temperature 72°C, and a final indefinite hold step of 4°C.

PCR products were quantified by 2% agarose gel electrophoresis and product size was confirmed through a visual comparison with Sigma Direct load Wide Range DNA marker (Sigma-Aldrich Denmark A/S).

Direct sequencing. PCR products were purified using ExoSap (Affymetrix, Santa Clara, CA, USA) and sequenced using the Big Dye dideoxy terminator cycle sequencing kit (Applied Biosystems) and the 3730 DNA Analyzer (Applied Biosystems). Sequence analysis was carried out using the Sequencher 4.9 software (Gene Codes Corporation, Ann Arbor, MI, USA)

Data analysis

Multiple sequence alignment and nucleotide conservation across species. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analyses of the two miRNA genes which harboured nucleotide variants (*miR-1-2* – NR_029662.1 and *miR-133a-2* – NR_029676.1) identified several similar sequences in a number of species. These sequences were aligned in Bioedit using ClustalW in order to identify conserved regions [49].

Prediction of RNA secondary structure. RNAfold Webserver (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) was used to compute the minimum free energy (MFE), the partition function, the matrix of base pairing probabilities, and the centroid structure [50].

Statistical analysis

Tests for allele frequencies, heterozygosity values, genic differentiation and Hardy-Weinberg equilibrium were carried out using the exact tests of GenePop on the web (http://genepop.curtin.edu.au/). Fisher's exact test was used to detect differences in allele distribution with respect to mutation status; a 5% alpha error was considered acceptable. The exact Hardy Weinberg test was applied to compare the overall frequencies of the identified genotypes and verify whether the distribution of these genotypes was in Hardy-Weinberg equilibrium [51,52].

Results

Three genetic variants were identified in this cohort, minor allele frequencies (MAF) for mutation carriers, non-mutation carriers and in silico controls are reported in Table II. All variants conformed to Hardy Weinberg expectations and there was no statistically significant difference in allele frequencies between mutation carriers ($n = 48$) and non-mutation carriers ($n = 77$).

Table I. Gene information and primer sequences for *mir-1-1*, *mir-1-2*, *mir-133a-1* and *mir-133a-2* amplification.

NCBI ref seq	miRNA gene	Forward primer	Reverse primer	Amplicon
NR_029780.1	*mir-1-1* (71 bp)	ACACAGAGAGGGCTCCGGCA	ACACGACCGTCCACCAACGC	342 bp
NR_029662.1	*mir-1-2* (85 bp)	TTGCCAAAGGTCATCTGTTCATGACT	TGGAACCATTAATGCCATGCTTCAGG	365 bp
NR_029675.1	*mir-133a-1* (88 bp)	AGCGCAGGAAAACAGTAGGA	TTTGAAATCCTTAAGTCATCCATACA	444 bp
NR_029676.1	*mir-133a-2* (102 bp)	ATCTCCATCGGGACTGCTT	GGGCTTCACTTACTTGGAGCT	264 bp

Table II. Minor allele frequencies of the genetic variants identified in this study.

Gene	SNP ID	Variant	MAF study sub-populations		MAF dbSNP populations		
			LQTSmc ($n=48$)	LQTSnmc ($n=77$)	HapMap-CEU ($n=113$)	pilot1_CEU_low_coverage_panel ($n=60$)	CSAgilent ($n=247$)
miR-1-2	rs9989532	n.100A > G	0.042	0.032	0.013	0.008	
miR-133a-2	rs13040413	n.−19G > A	0.250	0.312		0.250	
	rs200375711	n.98C > T	0.000	0.006			0.002

MAF, minor allele frequency; mc, mutation carrier; nmc, non-mutation carrier. All variants were in Hardy Weinberg Equilibrium, allele distribution was not significantly different between the two sub-populations.

No genetic variants were identified in *miR-1-1*; but in *miR-1-2* we identified a single substitution (rs9989532: *n.100A> G*) (Figure 1); in nine probands (all heterozygote carriers). Multi-species sequence comparison of DNA sequences similar to the miR-1-2 gene region is represented in Figure 2; the *n.100G* nucleotide is highly conserved among all mammals queried as well as zebrafish in a region which is highly conserved among apes.

No sequence variants were detected in *miR-133a-1*, but in *miR-133a-2* we identified two substitutions (*n.−19G> A* and *n.98C> T*) (Figure 1). The *n.−19G> A* polymorphism occurred in 64 probands (eight homozygote carriers and 56 heterozygote carriers), while the *n.98C> T* variant occurred in a single non-mutation carrier, Similar allele frequencies are reported in dbSNP for these variants; rs13040413, *n.−19G> A* has a reported MAF of 0.250 in the CEU population and rs200375711, *n.98C> T* has a reported MAF of 0.002 in the ClinSeq population (CSAgilent), a cohort of primarily Caucasian, atherosclerotic heart patients [53] (Figure 1, Table II). Multi-species sequence comparison of DNA sequences similar to the *miR-133a-2* gene region is represented in Figure 3; the *n.−19G* nucleotide is not conserved, however the *n.98C* nucleotide is highly conserved among all mammals queried as well as chicken and zebrafish; the region surrounding *n.98C* is highly conserved among apes.

Discussion

Presently, mutations in 13 genes are described to cause LQTS; taken together mutations in these genes explain 50–70% of LQTS cases. This means that causality in 30–50% of LQTS cases is as yet unknown. Animal models suggest that miRNAs might be involved in the regulation of cardiac action potential [35].

A number of associations between SNPs in predicted miRNA binding sites on target mRNAs and phenotypic traits have been reported [22,54–56]. A limitation of this study is that we have not assessed the MiR-1 and MiR-133A binding sites of putative target transcripts; instead we focused on identifying genetic variation within the miRNA genes themselves.

SNPs are rarely seen in the seed regions (short 3–8 nucleotide regions important for target specificity) of miRNAs; which suggests a strong selective constraint on the seed regions of mature miRNA. However, Mencia et al provided the first example of

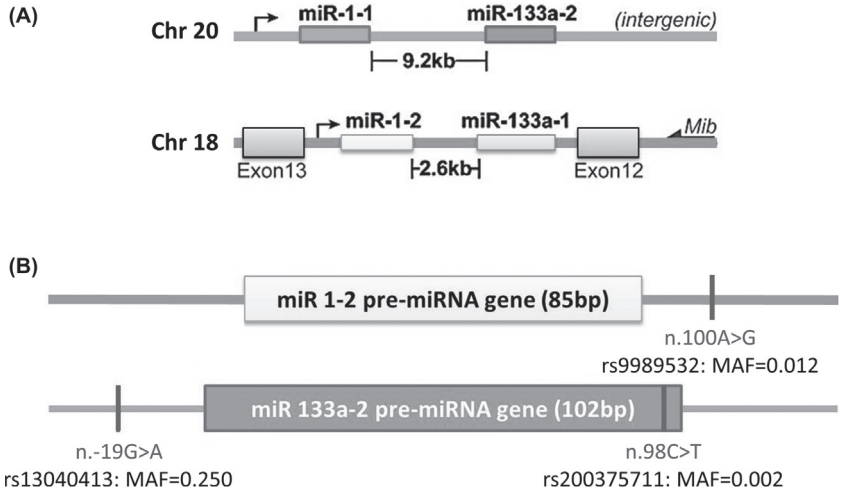

Figure 1. (A) Genomic structure of the miR-1 and miR-133 clusters. (B) Genomic structure of *mir-1-2* and *mir-133a-2*, variants identified in this study are represented by a red line. The minor allele frequencies (MAF) indicated here are representative of the CEU population as reported in 1000 genomes [60], except rs200375711 (*mir-133a-2:n.98C> T*) which was not identified in 1000 genomes but was identified in one of 493 atherosclerosis patients of European descent from the ClinSeq whole-exome sequencing project [53]. This Figure is reproduced in colour in the online version of *The Scandinavian Journal of Clinical & Laboratory Investigation*.

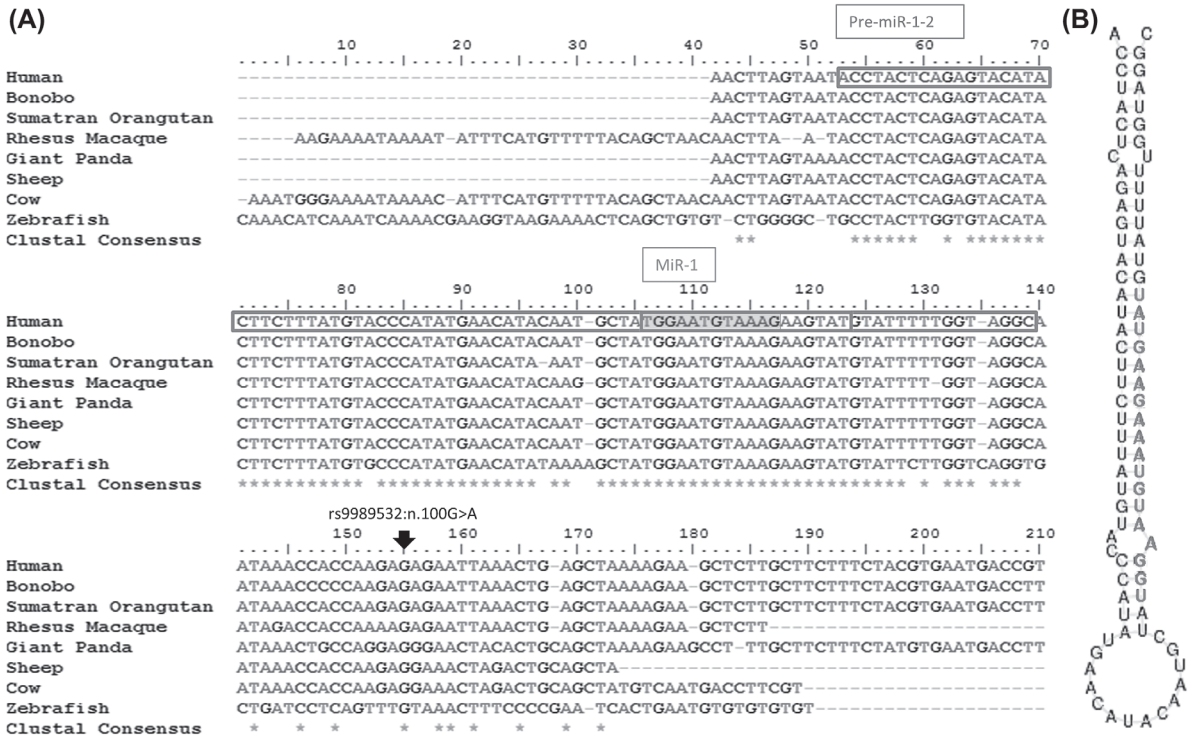

Figure 2. (A) Pri-miR-1-2 multiple species sequence alignment. Pre-miR-1-2 is indicated in a blue box, mature MiR-1 is indicated in a red box, the seed region is highlighted, rs9989532 is indicated by a black arrow. (B) Pre-miR-1-2 secondary structure, mature MiR-1 is indicated in red. Secondary structure was predicted using RNAfold Web Server [50]. This Figure is reproduced in colour in the online version of *The Scandinavian Journal of Clinical & Laboratory Investigation.*

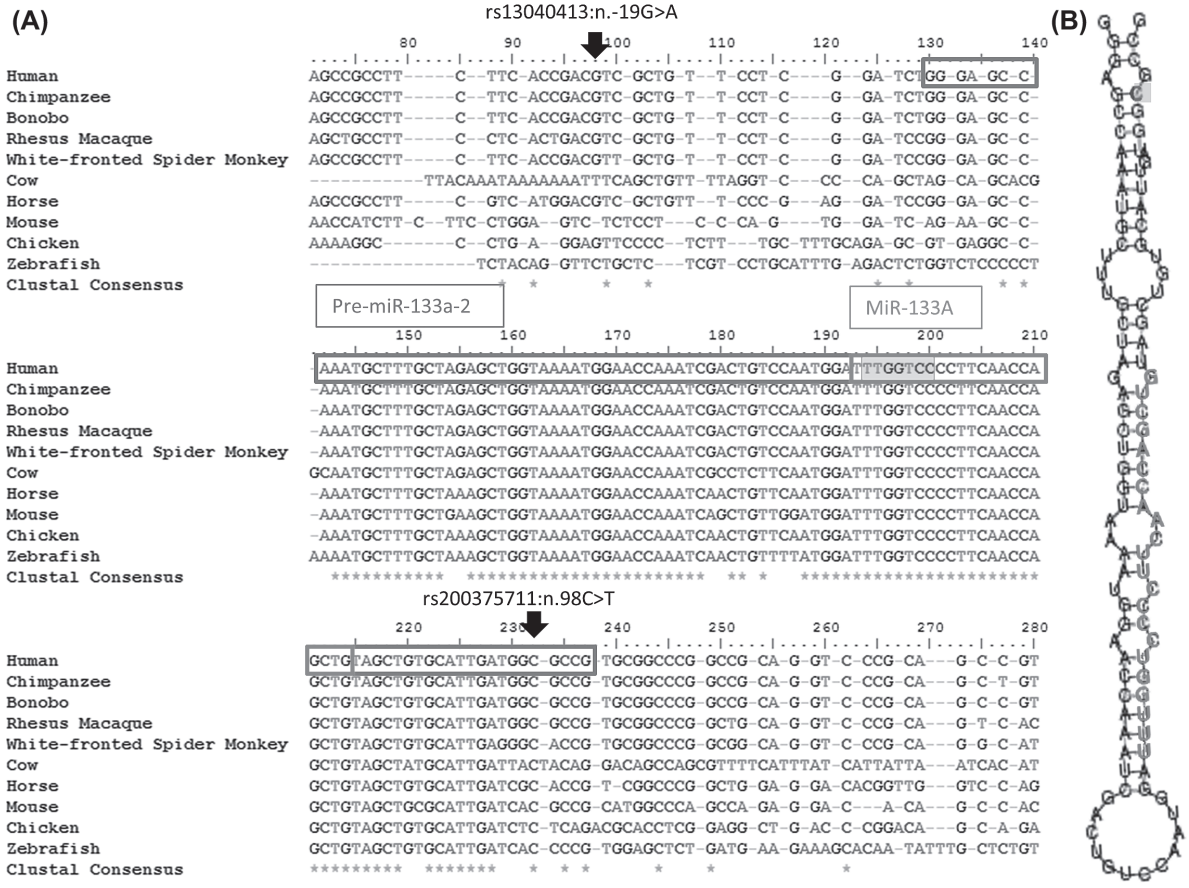

Figure 3. (A) Pri-miR-133a-2 multiple species sequence alignment. Pre-miR-133a-2 is indicated in a blue box, mature MiR-133A is indicated in a red box, the seed region is highlighted, rs13040413 and rs200375711 are indicated by black arrows. (B) Pre-miR-133a-2 secondary structure, mature MiR-133A is indicated in red, n.98C is indicated in blue. Secondary structure was predicted using RNAfold Web Server [50]. This Figure is reproduced in colour in the online version of *The Scandinavian Journal of Clinical & Laboratory Investigation.*

human inherited condition associated with miRNA mutations; when they discovered that mutations in the seed region of MiR-96 were responsible for non-syndromic progressive hearing loss in two families [57]. Outside of the seed region several variants have been associated with clinical phenotypes. Dorn et al. demonstrated that a rare variant in mature MiR-499 protected against cardiomyopathy in a transgenic mouse model [58] and Ohanian et al. identified a genetic variant in *mir-133a-2* which altered strand abundance resulting in an accumulation of the miRNA* strand in an atrial fibrillation patient [59]. Furthermore, several SNPs in pri-miRNA genes have been reported to affect processing and expression levels of mature miRNA [52].

Conclusion

To the best of our knowledge, this is the first study to report miRNA genetic variation in LQTS patients. We found three allelic variations none of which affect the mature miRNA products, although the *miR-133a-2:n.98C> T* variant is present in the excised pre-miRNA molecule (Figure 1) and may affect subsequent processing to mature MiR133A. Our findings indicate that sequence variation of *miR1-1*, *miR1-2*, *miR133a-1* and *miR133a-2* are not a cause of LQTS in this cohort.

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Declaration of interest: The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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