MicroRNAs in cardiac diseases: The devil is in the details
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CHAPTER 7

FUNCTIONAL VARIANTS IN THE 3'UTR OF KCNQ1 CREATE BINDING SITES FOR MIRNA-378: AN EXPLANATION FOR THE ALLELE-SPECIFIC EFFECTS ON DISEASE SEVERITY IN TYPE 1 LONG QT SYNDROME

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ABSTRACT

A mutation in one copy of the KCNQ1 gene causes type 1 long QT syndrome, a disease characterized by prolonged QT interval and life-threatening arrhythmias. It is unclear why in family members carrying exactly the same mutation disease severity varies greatly. We hypothesized that this variation is caused by differences in the balance in expression between the healthy and mutated allele, which can be caused by variants in the 3'UTR.

Indeed, in a previous study we found that 50% of the family members carried variants in the 3'UTR of KCNQ1 that suppress translation of the allele they are residing on. When these variants resided on the mutated allele, this suppressed formation of the mutated protein and decreased QT duration and the occurrence of symptoms. This novel mechanism largely explained the variation in disease severity in the studied populations.

Here we further show by allele-specific real-time PCR and western blot that these suppressive variants are able to reduce expression of endogenous KCNQ1 mRNA and protein levels in an allele-specific manner. We also show that these variants create a stronger binding site for miRNA-378, which allows miR-378 to suppress that specific allele. Furthermore, we show the crucial role of miR-378 in the suppressive effects of the SNPs by inhibition of this miRNA in cultured cardiomyocytes. This creates the possibility to inhibit miR-378 as a novel therapy based on individual genomic variants to shift the allelic balance from the mutant to the healthy protein in the most severely affected patients that carry the suppressive variants on their healthy allele.
INTRODUCTION

Long QT syndrome (LQT) is the most common hereditary cardiac arrhythmia, characterized by abnormal QT-interval prolongation on the surface ECG. LQT type 1 (LQT1) is the most prevalent form of LQT with a prevalence of 1 in 3000 individuals. LQT1 is an autosomal dominant disease caused by loss-of-function mutations in KCNQ1. These mutations reduce $I_{Ks}$ current and thereby delay repolarization of cardiomyocytes, visible as QT prolongation on the ECG. This QT prolongation may result in life-threatening arrhythmias, that lead to syncope and even sudden death.

Since KCNQ1 mutations are usually dominant-negative, one would expect that each mutation carrier has a similar degree of LQT1. However, arrhythmias in family members with an identical mutation vary greatly in severity. We recently identified a novel mechanism that partially explains this high variability in LQT1 disease severity (Chapter 6). We hypothesized that this variation in severity is caused by imbalance in expression between the healthy and mutated allele, which predicts that QT prolongation is less severe when the mutated allele is expressed less, and vice versa. We identified three single nucleotide polymorphisms (SNPs) in the 3'UTR of KCNQ1 (rs2519184, rs8234, rs10798) of which the derived variant represses translation of the allele it is residing on. When these suppressive variants reside on the patient's healthy KCNQ1 allele, they suppress expression of this healthy allele, which results in a shift towards more mutated channels. This was seen as a significant increase in heart rate corrected QT-interval (QTc) and occurrence of symptoms (syncope, torsades de pointes, ventricular tachycardia/fibrillation, sudden death) compared to patients lacking these variants. The opposite was also true: when the suppressive variants resided on the mutant KCNQ1 allele, QTc was significantly shorter with less symptoms. We confirmed the translational repression by the variants in in vitro luciferase assays, where the variants suppressed translation of the luciferase reporter both in cultured cardiomyocytes and cardiomyocyte-derived H10-cells. However, it was unclear how these suppressive variants reduced expression of KCNQ1.

Here we show by allele-specific real-time PCR (as-qPCR) that these suppressive variants reduced expression of KCNQ1 mRNA in the left ventricle in an allele-specific manner. We further show that these allele-specific effects on mRNA expression result in a decreased KCNQ1 protein level in the left ventricle of healthy subjects heterozygous for the three SNPs.

We next tested the hypothesis that these variants in the 3'-UTR create a microRNA (miRNA) binding site to suppress translation of KCNQ1. In an in silico analysis we identified 28 miRNAs that were predicted to bind stronger to the 3'UTR containing the suppressive variants. Therefore, we screened all 28 miRNAs in luciferase assays to address which of these 28 miRNAs were able to suppress luciferase activity of the suppressive variants as compared to the non-suppressive variants. We found that miR-378 potently suppressed the luciferase signal in the presence of only the suppressive variants. Furthermore, we show the crucial role of miR-378 in the suppressive effects of the SNPs by inhibition of this miRNA in cultured cardiomyocytes.
These results suggest that binding of miR-378 to the variants may largely explain the disease severity in LQT1 patients with a KCNQ1 mutation. This further illuminates the therapeutic potential of miR-378 inhibition specifically in patients with the suppressive variants on their normal allele, which are the most severely affected patients, to restore the balance between their mutant and normal alleles.

METHODS

In Silico Analyses of Interactions Between miRNAs and KCNQ1 3'UTR SNPs

The KCNQ1 3'UTR mRNA sequence was retrieved from the Ensemble Genome Browser (NCBI build 36, hg18) and the three SNPs (rs2519184, rs8234, rs10798) mapped on this sequence. A sequence centered on the suppressive SNP variant with 30 nucleotides flanking each side was assembled. The search by sequence option of www.mirbase.org was used to identify potential miRNA binding sites within these sequences using an E-value cut-off of 1000. This algorithm is designed to find miRNA homologs and searches for miRNAs minimally matching 7 nucleotides within these sequences and their complementary strand. Out of all these miRNAs we selected the miRNAs with a human homolog binding to the strand that contains the 3'UTR sequence. MiRNAs that contained an additional nucleotide match in the seed sequence created by the suppressive variant of the SNP and miRNAs highly expressed in the heart that contained an additional nucleotide match outside the seed sequence were selected for further in vitro studies.

Human tissue collection

Human left ventricular free wall and septal myocardial samples were obtained according to protocols approved by the Mayo Foundation Institutional Review Board from decedents for which a consented autopsy was conducted and the decedent was deemed to have died from non-cardiac causes (e.g. trauma, cancer not involving the heart, etc.) following a standard post-mortem examination.

Genomic DNA was extracted from frozen myocardial necropsy tissue using the Qiagen DNAeasy Tissue Kit (Qiagen) and KCNQ1's 3'UTR of each specimen was genotyped. Therefore three amplicons spanning the final exon and the 3'UTR of KCNQ1 were amplified by PCR using primer pairs: 5'-GGCACCTTCCCTTCTCTGG-3' and 5'-ACCACCATGCCAGTGATGTC-3'; 5'-CACAGCCTGCACTTGGG-3' and 5'-CAGGGCTCCTCTCCAGC-3'; 5'-CAGTCTCACCATTTCCCCAG-3' and 5'-GCCCAAGACAGGAGCGAC-3'. Amplicons were sequenced using BigDye Terminator 3.1 (Applied Biosystems) chemistry.

Animal experiments

C57BL/6jOlHsd (Harlan) mice at 8 weeks of age were injected intravenously with 5 mg/kg antimiRs (Ribotask, n=10 per antimiR) at two different timepoints (day 0 and day 4). AntimiRs consisted of locked nucleic acid (LNA) bases and 2'-O-methyl RNA bases in a ratio 1:2, where all nucleotides were phosphorothioated. The antimiRs were designed to perfectly complement the
complete mature miR-378 sequence or to a negative control sequence.

At day 7, osmotic minipumps (Alzet) containing isoproterenol (60 mg/kg/day) were placed subcutaneously at the back of half of the mice (resulting in n=5 per group). Therefore, mice were sedated with 4% isoflurane and anesthesia was maintained by a mixture of O₂ and 2.5% isoflurane.

One week after placing of the minipumps, we measured left ventricular (LV) function and dimensions by transthoracic two-dimensional echocardiography using a Vevo 770 Ultrasound (Visual Sonics) equipped with a 30-MHz linear array transducer. Mice were sedated with 4% isoflurane and anesthesia was maintained by a mixture of O₂ and 2.5% isoflurane. M-mode tracings in parasternal short axis view at the height of the papillary muscle were used to measure LV internal diameter at end-systole and end-diastole. Fractional shortening was calculated from these internal diameters using the following formula: ((LV end-diastolic diameter - LV end-systolic diameter) / LV end-diastolic diameter) x 100%. After echocardiography, mice were sacrificed. Immediately after euthanization, hearts were injected with 1 mol/L KCl to induce relaxation of cardiac myocytes, fluid was removed from the lumen and hearts were weighed. LV tissue was snap-frozen in liquid nitrogen en stored at -80ºC.

**Plasmid Construction**

Using polymerase chain reaction, luciferase reporter plasmids were constructed by amplifying the 3’UTR of KCNQ1 from a patient heterozygous for the SNPs rs2519184, rs8234 and rs10798 with the primers: 5’-ACTGACTAGTCATGGACCATGCTGTCTG-3’ and 5’-ACTGGAGCTCCAGCCTGTGATTCTCCACG-3’. This 878 base pairs fragment was cloned into the pMIR-REPORT™ Luciferase vector (Ambion) downstream of the luciferase coding region, creating luciferase-KCNQ1-3’UTR-GAA (non-suppressive haplotype) and luciferase-KCNQ1-3’UTR-AGG (suppressive haplotype).

The miRNA overexpression vectors were constructed by PCR-amplification of the miRNA precursor DNA form human genomic DNA using the primers in Table 1. These fragments were cloned into the pCDH1-MCS1-EF1-Puro vector (System Biosciences) under the control of a CMV promoter. The negative control miRNA overexpression vector PCDH1-control-miRNA is based on the pcDNATM6.2-GW/miR-neg control plasmid (Invitrogen) which contains an insert that can form a hairpin structure that is processed into mature miRNA but is predicted not to target any known vertebrate gene. All generated constructs were verified by sequencing.

**Cell culture and transfections**

COS7-cells were cultured under standard conditions. Cells were plated in 24-wells plates at a density of 1.2*10⁵ cells per plate for transfections and transiently transfected with per well 5 ng Renilla luciferase plasmid, pHRL vector (Promega), 100 ng of either luciferase-KCNQ1-3’UTR-GAA or luciferase-KCNQ1-3’UTR-AGG, and 25 or 100 ng of miRNA overexpression construct with empty pCDH1 as filler using polyethyleneimine (PEI). The miRNA overexpression constructs (1 μg) were also transiently transfected with PEI into COS7-cells plated in 6-wells plates to confirm
Table I. Primer sequences used for cloning of overexpression vectors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (restriction sites underlined)</th>
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| Pri-miR-133b | Fw 5'-ACTGGAAATTCTCGAGTGGTTAGCTCAGGGTAATG-3'  
Rv 5'-ACTGGCGGCCGCTATGATCTCTTGTGAACCTGGG-3' |
| Pri-miR-27a  | Fw 5'-ACTGGAAATTCTGCCACCGAGGAGATC-3'  
Rv 5'-ACTGGGATCCAGGATGGCAGGCAGACAGG-3' |
| Pri-miR-193b | Fw 5'-ACTGGAAATTCTAAACCCAGTAACGATTCC-3'  
Rv 5'-ACTGGGATCCACTTATAAGAATACCGCCCTC-3' |
| Pri-miR-328  | Fw 5'-ACTGGAAATTCTGGTGGACACGTCTGTGCTC-3'  
Rv 5'-ACTGGGATCCAGGCGTCATCGCTTGGTCTTG-3' |
| Pri-miR-631  | Fw 5'-ACTGGAAATTCTGGAGGATGGATGAGAAACGAG-3'  
Rv 5'-ACTGGCGGCCGCACAGCCTAAGGCGGTGAG-3' |
| Pri-miR-663  | Fw 5'-ACTGGAAATTCTGGAGGATGGATGAGAAACGAG-3'  
Rv 5'-ACTGGCGGCCGCACAGCCTAAGGCGGTGAG-3' |
| Pri-miR-1183 | Fw 5'-ACTGGAAATTCTCTACCTGTCTTACCTACC-3'  
Rv 5'-ACTGGGATCCATTATGCAGACCTAGCTCC-3' |
| Pri-miR-1204 | Fw 5'-ACTGGAAATTCTGGTGGACACGTCTGTGCTC-3'  
Rv 5'-ACTGGGATCCAGGCGTCATCGCTTGGTCTTG-3' |
| Pri-miR-1909 | Fw 5'-ACTGGAAATTCTGGTGGACACGTCTGTGCTC-3'  
Rv 5'-ACTGGGATCCAGGCGTCATCGCTTGGTCTTG-3' |
| Pri-miR-378  | Fw 5'-ACTGGAAATTCTGGTGGACACGTCTGTGCTC-3'  
Rv 5'-ACTGGGATCCAGGCGTCATCGCTTGGTCTTG-3' |
| Pri-miR-711  | Fw 5'-ACTGGAAATTCTGGTGGACACGTCTGTGCTC-3'  
Rv 5'-ACTGGGATCCAGGCGTCATCGCTTGGTCTTG-3' |
| Pri-miR-29c  | Fw 5'-ACTGGAAATTCTGGTGGACACGTCTGTGCTC-3'  
Rv 5'-ACTGGGATCCAGGCGTCATCGCTTGGTCTTG-3' |
overexpression. Furthermore, COS7-cells plated at a density of 1.2*10^6 in 24-wells plates were transiently transfected using PEI with 200 ng luciferase-KCNQ1-3'UTR-GAA and luciferase-KCNQ1-3'UTR-AGG in different ratios to optimize the allele-specific Q-PCR.

Cardiomyocytes from 1-2-day-old Lewis neonatal rats were isolated and cultured as described previously. Neonatal rat cardiomyocytes plated at a density of 250,000 cells per well in 24-wells plates were transfected using lipofectamine 2000 reagent (Invitrogen) with 50ng CMV-β-galactosidase vector, 100ng of either luciferase-KCNQ1-3'UTR-GAA or luciferase-KCNQ1-3'UTR-AGG, and 125 pmol antimiR-378 or antimiR-54 (Ribotask), a c.elegans miRNA not expressed in mammals. To confirm inhibition of miR-378 by antimiR-378, 500 pmol antimiR-378 was transfected into COS7-cells plated at a density of 1.2 *10^6 cells per plate in 6-wells plates using lipofectamine.

### Luciferase assays

At 48 hours after transfection, cells were lysed and assayed for luciferase, Renilla luciferase, and β-galactosidase activity with a luminometer (Glomax multi detection system, Promega). Renilla luciferase or β-galactosidase activity was assayed to normalize luciferase results for cell densities and transfection efficiency.

### RNA isolation

Total RNA from myocardial tissue and cultured cells (48 hours after transfections) was isolated using Trizol (Invitrogen) according to the manufacturer's protocol.

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<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (restriction sites underlined)</th>
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| Pri-miR-125a | Fw 5’-ACTGGAATCTGTTCTTTACAGTGAGGT-3’  
Rv 5’-ACTGGGATCCGATGCTGCTGTCAGCT-3’ |
| Pri-miR-148  | Fw 5’-ACTGGAATTCTCCATTATCGGTGCT-3’  
Rv 5’-ACTGGGATCCAGTCTTAGGTCAGTGACATGC-3’ |
| Pri-miR-220a | Fw 5’-ACTGGAATTCACAGGCAGGTGGTGACTCTG-3’  
Rv 5’-ACTGGGATCCACAGAGCTGGTAGGCACTGTC-3’ |
| Pri-miR-342  | Fw 5’-ACTGGAATTCAGAGAGACTGACACATCAGAGG-3’  
Rv 5’-ACTGGGATCCAGTCTTAGGTCAGTGACATGCC-3’ |
| Pri-miR-362  | Fw 5’-ACTGGAATTCACAGGCAGGTGGTGACTCTG-3’  
Rv 5’-ACTGGGATCCACAGAGCTGGTAGGCACTGTC-3’ |
| Pri-miR-369  | Fw 5’-ACTGGAATTCACAGGCAGGTGGTGACTCTG-3’  
Rv 5’-ACTGGGATCCACAGAGCTGGTAGGCACTGTC-3’ |
| Pri-miR-377  | Fw 5’-ACTGGAATTCACAGGCAGGTGGTGACTCTG-3’  
Rv 5’-ACTGGGATCCACAGAGCTGGTAGGCACTGTC-3’ |
| Pri-miR-1228 | Fw 5’-ACTGGAATTCACAGGCAGGTGGTGACTCTG-3’  
Rv 5’-ACTGGGATCCACAGAGCTGGTAGGCACTGTC-3’ |
Real-time PCR

For as-qPCR 400ng of input RNA was reverse transcribed using superscript II Reverse transcriptase (Invitrogen) with oligo-dT primers according to the manufacturer’s protocol. The real-time PCR was performed using LightCycler 480 SYBR green master I (Roche). For SNP rs8234 we used an allele-specific reverse primer (5’-ACCACAAATTATTGATTCTATCGAT/C-3’) and a general forward primer (5’-AGCCAGCCAAACACACAG-3’). Real-time PCR reactions were performed on a lightCycler480 system II (Roche) using the following program: 5 minutes pre-incubation at 95ºC and 40 cycli of 10 seconds denaturation at 95ºC, 20 seconds annealing at 60ºC, and 20 seconds elongation at 72ºC. Data were analyzed using LinRegPCR quantitative PCR data analysis software. The starting concentration of transcripts estimated by this software were corrected for the estimated starting concentration of the housekeeping gene GAPDH (5’-ACCCACTCCTCCACCTTTGAC-3’ and 5’-ACCCTGTTGCTGTAGCCAAATT-3’) and for the estimated starting concentration of a total KCNQ1 amplicon closely resembling the allele-specific amplicon (5’-GAAGTGACGGTTCCTACAC-3’ and 5’-AGCTTGCACAATTAATAATCAAAATC-3’).

For miRNA detection 400 ng input RNA was reverse transcribed using the miScript reverse transcription kit (Qiagen) according to manufacturer’s protocol. Real-time PCR was performed using the miScript SYBR Green PCR kit (Qiagen). The reaction was performed according to manufacturer’s protocol with 4 times diluted cDNA. The cDNA was amplified in a lightcycler 480 system II (Roche) using the following program: 95°C for 15 minutes and 40 cycli of 97ºC for 45 seconds, 55ºC for 45 seconds, and 72ºC for 45 seconds. The used forward primer had the same sequence as the miRNA and the reverse primer was 5’-GAATCGAGCACCAGTTACGC-3’.

MiRNA expression was analyzed using linRegPCR and normalized for expression of U6 (5’-CTCGGTTCGCCAGACA-3’ and 5’-AACGCTTCACGAATTTGC-3’).

Western Blots

Cardiac lysates from autopsy hearts were electrophoresed on pre-cast 10% SDS-polyacrylamide gels (Bio-Rad), transferred to nitrocellulose membranes, and blotted with rabbit anti-KCNQ1 (Santa Cruz Biotechnology) and mouse anti-GAPDH (Millipore) primary antibodies. Blots were incubated with the appropriate goat anti-rabbit IgG (Santa Cruz Biotechnology) or rabbit anti-mouse IgG (Abcam) horseradish peroxidase (HRP)-conjugated secondary antibody and developed by enhanced chemiluminescence (GE Healthcare) to visualize protein bands. Band density was quantified using ImageJ software (http://rsbweb.nih.gov/ij/).

Statistical analysis

Values are expressed as mean ± standard error. Differences between groups were compared using analysis of variance (ANOVA) or Student’s t test, where appropriate and p-values <0.05 were considered statistically significant.
RESULTS

Variants in KCNQ1’s 3’UTR suppress endogenous KCNQ1 expression

In our previous study (chapter 6) we showed that the presence of three variants (SNPs rs2519184, rs8234 and rs10798) in the 3’UTR of KCNQ1 suppressed translation of a luciferase reporter gene in cultured cardiomyocytes and cardiomyocyte-derived H10-cells. However, it is unclear whether these suppressive variants also reduce endogenous KCNQ1 levels in the human myocardium. Therefore we selected myocardial samples of subjects who had died due to non-cardiac causes and compared KCNQ1 expression on mRNA level by allele-specific real-time PCR (as-qPCR) in subjects heterozygous for all three SNPs to homozygous subjects. The allele-specificity of this as-qPCR was based on rs8234 and confirmed in COS7-cells transfected with different ratios of the luciferase-KCNQ1-3’UTR-GAA and luciferase-KCNQ1-3’UTR-AGG constructs (Supplemental figure 1). In the myocardial subjects the specificity of the as-qPCR was further confirmed in the control group, homozygous for the non-suppressive GAA haplotype (Figure 1A). In this group, there was supposed to be no allele-specific amplification of the suppressive G variant of rs8234 (2nd SNP in the haplotype). We found that 3% of total KCNQ1 mRNA in these patients was detected by the allele-specific amplification of this G variant, indicating minor read-through of this amplification reaction. Despite this read-through we were able to detect differences in allele-specific expression in the group heterozygous for the 3 SNPs. In case the SNPs do not influence KCNQ1 mRNA expression we would expect a 50:50 ratio in amplicons derived from both alleles in this heterozygous group. We detected a 10% difference in expression of both alleles, which was the lowest for the allele with the suppressive variants. These results indicate that also endogenous KCNQ1 expression is allele-specifically inhibited by the suppressive variants of the three SNPs.

We further show by western blot that this suppression of KCNQ1 mRNA expression results in a reduction of Kv7.1 (Figure 1B), which is the protein potassium channel formed from KCNQ1 mRNA. Compared to subjects homozygous for the non-suppressive haplotype (GAA/GAA), patients homozygous for the non-suppressive variant of rs2519184 and heterozygous for rs8234 and rs10798 (GAA/AGG) showed a minor non-significant reduction in Kv7.1 protein levels, while patients heterozygous for all three SNPs (GAA/AGG) showed a strong significant reduction in Kv7.1 compared to both other groups. Together, these data indicate that in human myocardium the suppressive variants of SNPs rs2519184, rs8234 and rs10798 decrease the levels of mRNA stemming from the allele containing these variants, which results in lower levels of Kv7.1 protein.

Suppressive variants create binding sites for miR-378 to allele-specifically suppress KCNQ1 expression

The suppressive nature of these three SNPs in the 3’UTR of KCNQ1 suggests that these variants create a miRNA binding site. We took a bioinformatical approach to identify miRNAs predicted to bind stronger to the 3’UTR containing the suppressive variants. For this purpose we inspected
the 3’UTR of KCNQ1, in particular 30 nucleotides at both sides of the three SNPs. We entered these sequences as input in the miRbase algorithm. Because this algorithm is not designed for target site prediction, but to find miRNA homologs within given sequences it searched for miRNAs minimally matching 7 nucleotides within these sequences and their complementary strand. Out of all these miRNAs we selected the miRNAs with a human homolog binding to the strand that contains the 3’UTR sequence. We further selected the miRNAs for in vitro testing based on creation of an additional nucleotide match in the seed sequence of the miRNA or an additional nucleotide match outside the seed sequence for miRNAs highly expressed in the heart. This in silico analysis yielded a total of 28 candidate miRNAs predicted to bind stronger to rs2519184 (miR-27a*, miR-193b, miR-328, miR-367, miR-378, miR-631, miR-663b, miR-1183, miR-1204, miR-1909*), rs8234 (let-7b*, miR-16, miR-18b*, miR-26a-2*, miR-31*, miR-134, miR-137, miR-378, miR-422a, miR-711) or rs10798 (miR-29c, miR-125a-5p, miR-148*, miR-220a, miR-
342-3p, miR-362-3p, miR-369-5p, miR-377, miR-1228). We overexpressed all 28 miRNAs in COS7-cells and performed luciferase assays to investigate whether these miRNAs repressed luciferase activity of the luciferase-KCNQ1-3’UTR-AGG construct (suppressive variants) compared to the luciferase-KCNQ1-3’UTR-GAA construct (non-suppressive variants) (Figure 2). We also overexpressed miR-133b as a negative control miRNA, whose binding was not affected by the three SNPs. Most miRNAs, including miR-133b, showed a minor non-significant reduction in luciferase activity of the luciferase-KCNQ1-3’UTR-AGG (suppressive variants) construct. Four miRNAs (miR-422a, miR-29c, miR-220a, miR-369-5p) showed a significantly decreased luciferase activity of the luciferase-KCNQ1-3’UTR-AGG construct after overexpression of these miRNAs using 25 ng of the overexpression constructs, while this decrease was not significant after overexpression using 100 ng construct. Five miRNAs (miR-193b, miR-663b, miR-16, miR-18b*, miR-148*) showed a significantly decreased luciferase activity of the luciferase-KCNQ1-3’UTR-GAA construct containing the suppressive variants compared to the non-suppressive variants.

Figure 2. Several miRNAs reduced the activity of the luciferase reporter gene linked to the suppressive variants in a luciferase screen. We overexpressed the miRNAs using 25 and 100 ng of overexpression plasmid and determined whether these miRNAs were able to more strongly suppress the luciferase activity of the reporter construct containing the suppressive variants compared to the non-suppressive variants. 

miRNAs are ordered to the SNP that is predicted to strengthen their binding site, with the miRNAs of SNP rs2519184 in panel A, of SNP rs8234 in panel B, and of SNP rs10798 in panel C, where miR-133b is a miRNA which binding is not predicted to be affected by any of the SNPs. Panel D contains miR-378, which is the only miRNA predicted to bind stronger to both rs2519184 and rs8234. For this screen we used n=6 per condition and *p<0.05 compared to non-suppressive haplotype.
activity of the luciferase-KCNQ1-3'UTR-AGG construct only after miRNA overexpression by 100 ng of the overexpression constructs, indicating that a dose-response relationship might exist for these miRNAs. Three miRNAs (miR-378, miR-377, miR-711) showed a significantly decreased luciferase activity of the luciferase-KCNQ1-3'UTR-AGG construct after miRNA overexpression using both 25 and 100 ng of the overexpression constructs. We confirmed overexpression of these three miRNAs in COS7-cells by real-time PCR (Supplemental figure 2).

Strikingly, the predicted binding of one of these identified candidate miRNAs, miR-378, was altered by two of the three studied SNPs. The suppressive variants of both rs2519184 and rs8234 created an additional match in the seed sequence of miR-378 (Figure 3A). Furthermore, miR-378 is abundantly expressed in human myocardial samples at levels comparable to the cardiomyocyte-specific miR-208a (Figure 3B). Within the heart it is specifically expressed in cardiomyocytes.

Figure 3. MiR-378 is responsible for the suppressive effects of the derived variants. A) Binding of miR-378 to the 3'UTR of KCNQ1 is predicted to be stronger due to the suppressive variants (grey and underlined) of rs2519184 and rs8234, which create an additional binding nucleotide within the seed sequence of miR-378. B) MiR-378 is abundantly expressed in the myocardium (n=3) at levels comparable to the cardiomyocyte-specific miR-208a. C) Overexpression of miR-378 in COS7-cells reduces luciferase activity of the reporter construct with the suppressive variants compared to the non-suppressive variants (n=3 per condition). D) Inhibition of miR-378 in cultured cardiomyocytes abolished the suppressive effects of the variants on the luciferase activity (n=6 per condition). *p<0.05 compared to non-suppressive haplotype.
which indicates that this miRNA is co-expressed in the same cell-type as its created target KCNQ1 allowing targeting in vivo. We confirmed in another luciferase assay independent of the screen that miR-378 overexpression in COS7-cells (Supplemental figure 3A) repressed luciferase activity of the reporter containing the suppressive 3'UTR haplotype (AGG) compared to the non-suppressive haplotype (GAA), while overexpression of a negative control miRNA did not show any difference between the two reporter constructs (Figure 3C).

We next investigated whether presence of miR-378 in cardiomyocytes is necessary for the suppression of the luciferase construct containing the suppressive haplotype (AGG) we observed in our former study (chapter 6). Therefore we used specific LNA-based antimiRs directed against miR-378, which efficiently inhibit miR-378 expression (Supplemental figure 3B). The loss of miR-378 in cultured cardiomyocytes abolished the suppressive effects of the 3'UTR SNPs on the luciferase activity of the construct containing the suppressive haplotype (AGG), while this difference was still detected after transfection of a negative control antimiR (Figure 3D). Together, these data indicate that miR-378 is both sufficient and necessary for the specific suppression of the allele that contains the 3'UTR with the suppressive haplotype.

**Inhibition of miR-378 does not influence isoproterenol-induced hypertrophy.**

Above results suggest that inhibition of miR-378 may have therapeutic potential specifically in patients with the suppressive variants on their normal allele. In these patients, inhibition is expected to restore the balance between mutant and normal alleles. However, miR-378 is identified to be downregulated in rodent and human heart failure and therefore inhibition of miR-378 might induce or influence the cardiac remodeling process leading to heart failure. Therefore, we first investigated the effect of systemic inhibition of miR-378 using LNA-based antimiRs on the heart at baseline and after induction of hypertrophy by 1 week of isoproterenol infusion in mice. We show the loss of miR-378 in cardiac hypertrophy, as this miRNA is decreased in isoproterenol infused mice compared to control mice (Figure 4A). Furthermore, we confirm inhibition of miR-378 in anti-miR-378 injected mice compared to the anti-miR-NC injected mice (Figure 4A). As shown in Figure 4B, short-term (2 weeks) inhibition of miR-378 did not result in induction of cardiac hypertrophy at baseline or influence the development of hypertrophy induced by 1 week of isoproterenol infusion, as evidenced by similar increase in heart weight corrected for tibia length. Furthermore, this inhibition of miR-378 did not significantly influence cardiac function at baseline or after hypertrophy induction by isoproterenol infusion as measured by echocardiography (Figure 4C). These results indicate that loss of miR-378 is not harmful at the short-term and may be further evaluated as a potential therapeutic modality.

**DISCUSSION**

Our data indicate that SNPs in the 3'UTR of KCNQ1 suppress its expression. This is caused by a creation of stronger binding sites for miR-378 by the SNPs rs2519184 and rs8234, which allows...
Our study shows that miR-378 is at least partly responsible for the suppressive effects of the variants in vitro. Furthermore, stronger binding of miR-378 to rs2519184 and rs8234 might explain the clinical effects we observed (chapter 6), since rs8234 and rs10798 are in complete LD in our two populations, which indicates that rs10798 might not be functional. However, we cannot exclude that in vivo other mechanisms or additional microRNAs may play a role. In this regard, we identified other miRNAs in our luciferase screen that were able to repress the luciferase activity of the reporter construct with the suppressive variants. However, the abolishment of the suppressive effects of the variants in cardiomyocytes after inhibition of miR-378 indicates that in cardiomyocytes miR-378 is probably the most important miRNA to convey the suppression of the allele containing the suppressive variants. Furthermore, other miRNA-independent mechanisms might also influence the allelic imbalance and thus the disease severity in LQT1 patients. In this regard, SNPs at the 5’ side of KCNQ1 might influence its transcription in an allele-specific manner. GWAS studies recently revealed that intron 1 of KCNQ1 contains four SNPs associated with QTc duration in the general population.10-12 In addition, ChIP-seq revealed the presence of a putative enhancer in intron 1, as this region was occupied by
the enhancer-associated protein p300. These SNPs might thus influence enhancer activity and the resulting transcription of KCNQ1 in an allele-specific manner, which can also result in an imbalance between healthy and mutated protein expression and thus explain part of the variability in disease severity.

The involvement of miR-378 in the suppressive effects of the variants implies that factors influencing the expression of miR-378 might indirectly modify disease severity in LQT1 patients. This can be illustrated by LQT1 patients that carry the suppressive variants on their mutant allele. In these patients expression of the mutant allele is suppressed, which results in a shift towards less mutated channels and less severe LQT1. In the case that such a patient develops left ventricular hypertrophy or heart failure, this will result in a downregulation of miR-378 in their myocardium. Downregulation of miR-378 will partially release the suppression of the mutant allele, which will result in a shift towards more mutated channels and will increase severity of LQT1 in these patients.

Together, the results of our previous study and the results shown here imply that inhibition of miR-378 might be explored for its therapeutic potential in the most severely affected LQT1 patients. These patients carry the suppressive variants on their normal KCNQ1 allele. Inhibition of miR-378 would abolish the suppression of this normal allele, which will restore the balance in expression of mutant and normal allele, and therefore result in a decrease in severity of LQT1 disease. The fact that inhibition of miR-378 in patients with the opposite genotype would increase disease severity, due to release of suppression of the mutant allele, indicates that genetic testing in LQT1 patient should include analysis of the 3'UTR SNPs and determination of which allele contains the suppressive variants. In this regard, inhibition of miR-378 on the base of a personal genotype in LQT1 patients is a first step towards personalized medicine.

The use of systemic antimiR injections as therapeutic seems feasible, as inhibition of miR-122 in hepatitis C patients appeared safe and effective in the first clinical trial to antimiR-based therapeutics. However, systemic inhibition of miR-378 is expected not only to influence the allelic imbalance of KCNQ1 alleles, but might influence several other processes leading to side effects. Based on the current knowledge of the function of miR-378 several of these side effects might turn out positive. First, Carrer et al. generated a miR-378/miR-378* knockout mouse and show the resistance of these mice to high-fat diet induced obesity by an enhanced mitochondrial fatty acid metabolism and elevated oxidative capacity of insulin-target tissues. They propose inhibition of miR-378 as therapeutic in obesity and metabolic syndrome, indicating that weight loss might be a side effect when inhibition of miR-378 is used therapeutically in LQT1 patients. Second, miR-378 is found to enhance cell survival of U-87 glioblastoma cells in vitro and to promote tumor growth after injection of these cells into mice. This indicates that inhibition of miR-378 might also reduce tumor growth. Third, in contrast to the pro-survival effects of miR-378 on tumor cells, miR-378 is found to increase sensitivity of cultured cardiomyocytes to hypoxia-induced apoptosis, which indicates that inhibition of miR-378 might protect cardiomyocytes against apoptosis in coronary artery disease. However, opposite effects...
were also detected. H9c2 cardiomyocytes showed increased hypoxia-induced apoptosis after inhibition of miR-378. Furthermore miR-378 is found to inhibit phenylephrine (PE) induced hypertrophy in cultured cardiomyocytes, whereas inhibition of miR-378 resulted in increased hypertrophy at baseline and after PE stimulation. In addition, compensation for loss of miR-378 after transverse aorta constriction (TAC) in mice by AAV9-mediated expression of miR-378 attenuated cardiac hypertrophy and improved cardiac function. This indicates that inhibition of miR-378 might induce hypertrophy at baseline or aggravate the hypertrophic response to stress. We investigated the effect of inhibition of miR-378 at heart weight and cardiac function at baseline and after hypertrophy induced by 1 week of isoproterenol infusion and did not detect any effects of inhibition of miR-378 at these parameters in this short term experiment. It would be interesting to investigate the effects of inhibition of miR-378 on cardiac hypertrophy and cardiomyocyte apoptosis at the long term or after more severe stress by for example TAC or myocardial infarction models.

In conclusion, we show that the suppressive variants of the previously identified SNPs in KCNQ1’s 3’UTR suppress expression of the allele they are residing on by creating two functional binding sites for miR-378. This creates the possibility to inhibit miR-378 as a novel therapy based on individual genomic variants to shift the allelic balance from the mutant to the healthy protein.
REFERENCES


Supplemental figure 1. Confirmation of specificity of the allele-specific real-time PCR. COS7 cells were transfected with different ratios of the reporter constructs containing the non-suppressive (GAA) and suppressive (AGG) haplotype (n=2). Real-time PCR were specificity was based on rs8234 (2nd SNP in the haplotype) detected the specific amplicons in ratios comparable to the ratio of transfection, confirming the allele-specificity of this PCR.

Supplemental figure 2. Confirmation of miRNA overexpression in the luciferase screen. Overexpression of the 3 candidate miRNAs was confirmed by real-time PCR in COS7 cells (n=3). We show the relative expression compared to the negative control miRNA transfected.
Supplemental figure 3. Confirmation of overexpression and inhibition of miR-378. Transfection of the overexpression construct (A) and the antimiR-378 (B) in COS7-cells resulted in overexpression and inhibition of miR-378 respectively (n=3).