

RNA interference-based therapeutics for inherited long QT syndrome (Review)

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Abstract. Inherited long QT syndrome (LQTS) is an electrical heart disorder that manifests with syncope, seizures, and increased risk of torsades de pointes and sudden cardiac death. Dominant-negative current suppression is a mechanism by which pathogenic proteins disrupt the function of ion channels in inherited LQTS. However, current approaches for the management of inherited LQTS are inadequate. RNA interference (RNAi) is a powerful technique that is able to suppress or silence the expression of mutant genes. RNAi may be harnessed to knock out mRNAs that code for toxic proteins, and has been increasingly recognized as a potential therapeutic intervention for a range of conditions. The present study reviews the literature for RNAi-based therapeutics in the treatment of inherited LQTS. Furthermore, this review discusses the combined use of RNAi with the emerging technology of induced pluripotent stem cells for the treatment of inherited LQTS. In addition, key challenges that must be overcome prior to RNAi-based therapies becoming clinically applicable are addressed. In summary, RNAi-based therapy is potentially a powerful therapeutic intervention, although a number of difficulties remain unresolved.

Contents

1. Inherited long QT syndrome
2. Mechanisms underlying inherited LQTS
3. RNAi and its therapeutic potential for various cardiovascular diseases
4. RNAi-based therapeutic potential for LQTS

5. Limitations
6. Perspective and conclusion

1. Inherited long QT syndrome

Inherited long QT syndrome (LQTS) is an electrical heart disorder that is characterized by the abnormal prolongation of cardiac repolarization, which corresponds to the QT interval on an electrocardiogram. It has been observed that the prevalence of LQTS is ~1:2,500 among Caucasians (1). Clinically, patients with LQTS are at an increased risk of torsades de pointes, which manifests as palpitations, syncope, seizures and sudden cardiac death. To date, hundreds of mutations have been identified that contribute toward the development of LQTS, and these are present in at least 13 genes (Table I) (2-4). It has been determined that >90% of positive-genotype cases of LQTS exhibit one of three particular genotypes: LQT1, LQT2 and LQT3 (3,5-8).

Current treatment options for LQTS include the administration of β -adrenoceptor antagonists, the implantation of pacemakers or implantable cardioverter defibrillators (ICDs), and left cardiac sympathetic denervation (7). However, there are limitations associated with each of the current treatment options for LQTS (7-9). Firstly, ICDs are usually expensive and available only to a relatively small proportion of patients with LQTS. Drug-based therapies are widely available, but exhibit limited efficacy in patients with advanced LQTS. Furthermore, in blood relatives, LQTS symptoms may be severe, and generate complicated clinical situations. Although genomic studies have contributed significantly to the improvement of diagnostics and a limited number of treatments, the human genome is more complex than was initially expected. For example, individuals with identical genetic defects in the same pedigree may present with different characteristics. These differences may be partially due to the polygenic nature of genetic diseases, as there are multiple genetic factors that contribute toward the pathology of a disease, thus complicating the etiology of LQTS (10). Finally, the primary limitation of current therapeutic approaches is that their targets are relatively independent of the specific origin of inherited LQTS. To date, many mutations in various genes have been well-characterized and their toxic mutant protein products widely recognized as being responsible for the dominant-negative processes underlying the development of LQTS (11-14). However, for many of

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Table I. Inherited long QT syndrome.

Type	Affected gene	Chromosome	Protein	Current	Trigger	Prevalence (%)
LQT1	KCNQ1	11p15	I_{Ks} α -submit	$I_{Ks}\downarrow$	Exercise	42
LQT2	KCNH2	7q35-36	I_{Kr} α -submit	$I_{Kr}\downarrow$	Auditory, exercise, waking	45
LQT3	SCN5A	3p21-23	I_{Na} α -submit	$I_{Na}\uparrow$	Rest, sleep	8
LQT4	ANK2	4q25-27	Ankyrin-B	$I_{Ca}\uparrow$	Exercise	<1
LQT5	KCNE1	21p22	I_{Ks} β -submit	$I_{Ks}\downarrow$	Emotion, exercise	3
LQT6	KCNE2	21p22	I_{Kr} β -submit	$I_{Kr}\downarrow$	Rest, exercise	2
LQT7	KCNJ2	17p23-24	$I_{Kir2.1}$	$I_{K1}\downarrow$	Rest, exercise	<1
LQT8	CACNA1	12p13	I_{Ca} α -submit	$I_{Ca}\uparrow$	Exercise, anxiety	<1
LQT9	CAV3	3p25	Caveolin	$I_{Na}\uparrow$	Rest, sleep	rare
LQT10	SCN4B	11q23	NAV β 4	$I_{Na}\uparrow$	Exercise	N/A
LQT11	AKAP-9	7q21-22	Yotiao	$I_{Ks}\downarrow$	N/A	N/A
LQT12	SNTA1	20q11	α 1 syntrophin	$I_{Na}\uparrow$	N/A	N/A
LQT13	KCNJ5	11q24	Kir4.3	$I_{K-ACH}\downarrow$	N/A	N/A
JLN1	KCNQ1	11p15	I_{Ks} α -submit	$I_{Ks}\downarrow$	Emotion, exercise	1-7
JLN2	KCNE1	21p22	I_{Kr} β -submit	$I_{Ks}\downarrow$	Emotion, exercise	<1

LQT, long QT; JLN, Jervell and Lange-Nielsen; I_{Ks} , slowly activating delayed potassium rectifier current; I_{Kr} , rapidly activating delayed potassium rectifier current; I_{Na} , sodium current; I_{Ca} , calcium current; $I_{Kir2.1}$, inward rectifier potassium current; I_{K-ACH} , acetylcholine-activated potassium current; N/A, not available.

these culprit targets, there are currently no treatments available that successfully act on them. None of the current therapeutic approaches are able to differentiate a target consisting of a single-nucleotide polymorphism mutation. The inadequacies of current therapies have prompted the investigation and screening of novel alternative treatments for LQTS, including gene therapies. The process of RNA interference (RNAi) is increasingly recognized as serving a crucial function in regulating direct gene expression (15-19), and studies of the use of RNAi for modulating LQTS-associated gene function have been initiated (8,20).

2. Mechanisms underlying inherited LQTS

Mutations have been identified to cause ion channel dysfunction by various mechanisms. In this section, the human ether-à-go-go-related gene (hERG), whose frequency in mutation-identified LQTS patients is the highest in Chinese patients, is discussed as an example. hERG is in chromosome 7 and encodes the α -subunit of the rapidly activating delayed rectifier K^+ current (I_{Kr}) in the heart, a current that contributes toward the repolarization of the cardiac action potential (21,22). hERG potassium channels form homotetramers of identical six α -helical transmembrane-spanning domains (11,21,23,24). Delisle *et al* (12) and Gong *et al* (24) have reviewed a proposed classification system for LQT2 mutations, which organizes these mutations according to their underlying mechanisms. Class 1 mutations produce abnormal protein synthesis by defective transcription or translation. Class 2 mutations lead to defective protein trafficking. Class 3 mutations result in abnormal gating and/or kinetics. Class 4 mutations result in altered or absent channel selectivity or permeability. Class 5 mutations produce a reduction in mutant mRNAs through nonsense mediated

decay, thereby altering the quantity of mRNA available for subsequent hERG protein generation (11-14,24). The majority of hERG mutations exhibit loss-of-function phenotypes, due to haploinsufficiency or suppression of wild-type (WT) function by a dominant-negative mechanism. Dominant-negative current suppression is a mechanism by which mutant hERG alleles may lead to a reduction of the number of WT hERG channels in heterozygous patients, with the result that during the co-assembly of hERG WT and mutant hERG channel subunits, the tetrameric hERG channel complexes are rendered non-conducting. The dominant-negative suppressive effects of hERG mutations on WT function are the result of a number of defects (13,14). In the majority of human LQT2 mutations, the dominant-negative suppression of WT function is a result of the trafficking-deficient mechanism (12-14,25). The dominant-negative current suppression is explained most accurately as a conditional trafficking block imposed on WT channels by co-assembly with trafficking-deficient mutant subunits, with increased degradation as a consequence of impaired trafficking.

3. RNAi and its therapeutic potential for various cardiovascular diseases

RNAi may be generated by molecules other than mRNA and it may be possible to design and utilize RNAi-based techniques for numerous applications in molecular biology and medicine (15,17,18,26-29). RNAi is an evolutionary conserved mechanism for silencing sequence-specific post-transcriptional genes. RNAi was initially recognized by Fire *et al* (30) in 1998, who determined that double-stranded RNA was substantially more effective than a single strand at generating interference. Following injection into adult animals,

purified single strands of RNA exhibited a modest effect, whereas double-stranded mixtures resulted in marked and specific interference. The effects of this interference could be observed in the injected animals and their progeny. Only a small number of double-stranded RNA molecules were required per affected cell, indicating that stoichiometric interference with endogenous mRNA is unlikely and suggesting that there may be a catalytic or amplification component in the interference process (30). Currently, RNAi exhibits the potential to be a novel therapeutic strategy in various areas of medicine. The capability of RNAi to reduce the expression of pathological proteins is potentially applicable to virtually all classes of molecular target, including those that are difficult to selectively modulate with traditional pharmaceutical approaches involving small molecules or proteins (17,26-29,31-34). The exploitation of small RNA biogenesis and silencing gene pathways for heart disease, using small interfering (si)RNA against single targets or inhibiting the action of misregulated micro (mi)RNA, has yielded promising results and such agents are approaching clinical trials (17,26). Fitzgerald *et al* investigated the safety and efficacy of ALN-PCS, an siRNA that inhibits PCSK9 synthesis, in healthy volunteers with elevated levels of cholesterol who had received no lipid-lowering treatment (29). Administration of ALN-PCS led to a rapid, dose-dependent reduction in plasma PCSK9 levels, with higher doses exhibiting an extended duration of effect. The maximum reduction in low-density lipoprotein (LDL) cholesterol noted in an individual participant was 57%. These results suggested that the inhibition of PCSK9 synthesis by RNAi provides a potential alternative approach for reducing LDL cholesterol levels in healthy individuals with raised cholesterol. The study by Fitzgerald *et al* was the first to demonstrate the effective clinical application of RNAi-based therapeutic strategies against a validated endpoint (LDL cholesterol) in humans (29).

4. RNAi-based therapeutic potential for LQTS

In vitro heterologous system of human embryonic kidney (HEK)293 cells. RNAi provides a method for abolishing the alleles of genes that exhibit spontaneous or inherited polymorphisms and alternative splicing with single-point mutations identified in inherited disorders, genes that are critical for mediating the pathology of various diseases (8,27,35). Studies demonstrating the RNAi-mediated selective inhibition of mutant genes in dominant-negative genetic diseases led to a increased interest in RNAi technology as a potential therapeutic alternative for human inherited disorders (8,27,35). Recent studies have demonstrated the clinical potential of using RNAi in the treatment of LQTS (8,20). The dominant-negative mutation E637K-hERG results in the substitution of lysine for glutamic acid at position 637 in the pore-S6 loop transmembrane segment of hERG (20,36). The trafficking-deficient E637K-hERG disrupts WT-hERG channel biophysical properties. Lu *et al* (20) used RNAi to restore the properties of I_{Kr} in an *in vitro* heterologous system (HEK293) co-expressing WT-hERG and the LQT2 trafficking-deficient E637K-hERG mutant. WT-hERG localization in cells co-expressing the dominant-negative E637K-hERG mutant was restored to the membrane by siRNA. The specific siRNA-mediated inhibition of the E637K-hERG mutant restored the maximum and

tail current amplitudes. Similarly, siRNA treatment improved the kinetic properties of WT/E637K-hERG protein channels to a level comparable to that of WT-hERG protein channel. These observations initially indicated the potential of RNAi to inhibit E637K-hERG protein expression and protect against the dominant-negative effect of this mutation by restoring the kinetic properties of hERG protein channels (20).

In vitro induced pluripotent stem cells (iPSCs). Studies of RNAi-based therapeutics for LQTS continue to indicate novel directions for therapeutic intervention. Matsa *et al* (37) investigated the potential of RNAi-based therapeutics combined with the emerging technology of iPSCs. iPSCs are a type of pluripotent stem cell that may be generated directly from adult cells via the forced expression of transcription factors. iPSC technology has progressed rapidly since its initial development (38,39). iPSCs have been generated from a number of human cell lines, and novel approaches for improving the safety and efficiency of iPSCs have been suggested (40-44). iPSC technology provides a potential solution to the limitations of using heterologous cell lines or animals to model human diseases, particularly for investigating the consequences of human genetic variation on cellular phenotypes. Numerous groups have generated patient-specific iPSCs and differentiated them into relevant cell types, which continue to contain harmful mutations and manifest the morphological characteristics of the corresponding disease (40-42). Furthermore, a number of groups have reported the successful generation of iPSCs from inherited LQTS cells (Table II) (42-44). iPSCs derived from patients with LQTS may be differentiated into patient-specific iPSC-derived cardiomyocytes (iPS-CMs), offering a potentially unlimited source of materials for biomedical study. iPS-CMs may be used to recapitulate complex physiological phenotypes, probe toxicological testing and drug screening, clarify novel mechanistic insights and provide alternative strategies to rectify genetic defects at the cellular and molecular level.

Human iPS-CMs have been used to investigate the disease-inducing biophysical mechanisms of LQT2-associated mutation in a hERG *in vitro* model, to evaluate gene-based therapeutics for the treatment of inherited LQTS (38). Compared with the control iPS-CMs, LQT2 CMs exhibit significantly extended field potential duration (38). Treatment with E4031 caused arrhythmogenesis appearing as early after-depolarizations in LQT2 iPS-CMs but not in control myocytes, indicating increased sensitivity to I_{Kr} blockade. Further experiments investigating this putative disease-inducing biophysical mechanism indicated a dominant-negative phenotype due to the formation of non-functional WT/Mut tetramers with defective hERG membrane transport.

RNAi is a largely unexploited but increasingly well-studied intervention with the potential for use in a wide range of applications in the future (17,27,35,45). iPSCs genetically modified by RNAi exhibit practical applications for the improvement of cardiac function. In the study by Matsa *et al*, iPS-CMs reliably modeled disease pathologies *in vitro* and the benefits of allele-specific RNAi-based knockdown were assessed in these functional cells (38). When mutations are subjected to RNAi, the degradation of mutant mRNAs results before notable quantities of mutant protein are generated. By eliminating

Table II. Existing iPSC models of inherited long QT syndrome.

Syndrome	Species	Affected gene	Mutation	Somatic cells	Vector	Transcription factors	Methodology
LQT1	Human	KCNQ1	R190Q	Fibroblasts	Retrovirus	OCT3/4, SOX2, KLF4 and c-MYC	Whole-cell patch clamp, immunofluorescence
LQT2	Human	KCNH2 (hERG)	A614V	Fibroblasts	Retrovirus	SOX2, KLF4 and OCT4.	Whole-cell patch clamp, extracellular multielectrode recordings
LQT8	Human	CACNA1C	G406R	Fibroblasts	Retrovirus	SOX2, OCT3/4, KLF4 and c-MYC	Whole-cell patch clamp, calcium imaging
LQT2	Human	KCNH2 (hERG)	G1681A	Fibroblasts	Lentivirus	OCT4, NANOG, SOX2 and LIN28	Whole-cell patch clamp, extracellular multielectrode recordings
LQT2	Human	KCNH2 (hERG)	R176W	Fibroblasts	Retrovirus	OCT4, SOX2, KLF4 and MYC	Whole-cell patch clamp, extracellular multielectrode recordings
LQT1	Human	KNCQ1	1893delC (P631fs/33)	Fibroblasts	Lentivirus	OCT3/4, SOX2, KLF4 and c-MYC	Extracellular multielectrode recordings, patch-clamp analysis and immunostaining
LQT2	Human	KCNH2 (hERG)	G603D	T lymphocytes	Sendai virus	OCT3/4, SOX2, KLF4 and c-MYC	Immunocytochemistry
LQT3	Mouse	SCN5A	ΔKPQ	Fibroblasts	Retrovirus	Oct4, Sox2 and Klf4 or additionally with the fourth factor c-Myc	Patch-clamp technique, immunostainings
LQT2	Human	KCNH2 (hERG)	G1681A	Fibroblasts	Lentivirus	OCT4, NANOG, SOX2 and LIN28	Allele-specific RNA interference, whole-cell patch-clamp, multi-electrode array analysis
LQT2	Human	KCNH2 (hERG)	W1001X	Fibroblasts	Episomal	OCT4, SOX2, NANOG, LIN28, c-Myc and KLF4	Confocal imaging, voltage clamp

iPSC, induced pluripotent stem cell; LQT1, long QT syndrome type 1; KCNQ1, potassium voltage-gated channel, KQT-like subfamily, member 1; KCNH2, potassium voltage-gated channel, subfamily H (eag-related), member 2; hERG, human ether-à-go-go-related gene; LQT2, long QT syndrome type 2; LQT3, long QT syndrome type 3; SCN5A, sodium channel, voltage-gated, type V, α subunit.

abnormal mRNA transcripts, RNAi prevents the production of pathogenic proteins that result in deleterious effects on ion channels. A key physiological function of RNAi is to prevent severe disease phenotypes by converting the dominant-negative effect into haploinsufficiency. These observations indicate that further *in vivo* trials may be required in order to develop specific RNAi-based clinical treatments for a range of inherited cardiovascular and non-cardiovascular disorders induced by dominant-negative mutations.

5. Limitations

There are numerous problems to be solved prior to the use of RNAi therapy in clinical practice. To date, multiple barriers to achieving efficient and safe RNAi-based treatment have become evident, including specificity for the target gene, delivery to the correct cell or tissues, the duration of RNAi activity and the stability of the target mRNA and encoded protein (8,15-17,27,33,45,46). Efficient delivery to target cells and tissues is the primary challenge for RNAi-based therapeutics. A key issue is the physical guiding of the vector to the target tissue (33). Cardiac targeting has emerged as an extremely difficult task that has not been achievable in a simple and efficient manner using earlier gene transfer systems. A second key issue is the stability of the therapeutic vector. Small regulatory RNAs are inherently unstable, and viral vectors are currently the only available tools for non-topical *in vivo* therapy using these molecules. However, multiple proteins are also inherently unstable and/or intracellular, which similarly suggests the use of long-term stable vectors for long-term therapy (33). Third, the safety issues associated with RNAi-based therapies require consideration. Even assuming that the delivery problems can be solved, toxicity of small hairpin (sh)RNA may result from competition with the normal cellular miRNA processing system. shRNAs exert their therapeutic effects by mimicking this endogenous process and achieve silencing; however, they may disturb cellular miRNA pathways and thereby result in adverse effects. For other target diseases experimentally treated with cardiotropic RNAi vectors with low affinity to the liver, no hepatic or other toxicity has been observed (17), however toxicity remains a possibility. The off-target effects of regulatory RNAs are another possible source of toxicity and are difficult to assess (46). As a consequence, side-effects at the cellular level and *in vivo* require further investigation to assess the possible toxicity of the RNAi-based therapeutics.

6. Perspective and conclusion

Mutations associated with LQTS typically lead to trafficking deficiencies, which introduce dominant-negative effects and thus result in ion channel dysfunction. Recently, RNAi has emerged as a robust technology for improving disease phenotypes *in vitro* and in *in vivo* models of human disease. Early studies supported the potential new class of RNAi-based therapy in the treatment of numerous genetic diseases, as selective and potent gene expression inhibitors. The present review discusses examples of mutations that induce dominant-negative effects, which are able to destabilize mRNA transcripts via a mechanism known as RNAi in inherited LQTS. This mechanism helps to reduce the quantity

of mutant mRNA transcripts, thus reducing the production of toxic proteins. Although a number of studies are in progress, numerous challenges require solutions prior to the application of RNAi in clinical practice. Further studies are required to clarify the key obstacles, and to investigate strategies that may lead to safe and effective delivery technologies as RNAi-based therapies are adopted in clinical practice. Additionally, the combination of RNAi and certain emerging techniques, such as genetically-modified iPSCs to treat heart disease, are also promising. However, the field must progress further to enable the necessary development of this therapeutic approach for the treatment of patients with otherwise untreatable genetic diseases. Due to the pace of investigation, RNAi may emerge as a valuable therapy and has the potential to be used clinically for the treatment of LQTS.

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