KCNE3 Mutation V17M Identified in a Patient with Lone Atrial Fibrillation

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Abstract
Background: Atrial fibrillation (AF) is the most common cardiac rhythm disorder with a lifetime risk for development of 25% for people aged 40 or older [1]. In this study we aim for the functional assessment of a mutation in KCNE3 identified in a proband with early-onset lone AF. Methods: Screening of genomic DNA from the proband led to identification of a KCNE3 V17M missense mutation. We heterologously expressed the accessory channel subunit in Xenopus laevis oocytes together with its known interacting potassium channel α-subunits. Further, we applied RT-PCR on human total RNA from left and right atria and ventricle. Results: Electrophysiological recordings revealed an increased activity of Kv4.3/KCNE3 and Kv11.1/KCNE3 generated currents by the mutation, thereby conferring susceptibility of mutation carriers to faster cardiac action potential repolarization and thus vulnerability to re-entrant wavelets in the atria and thereby AF. Conclusion: Here we report a novel mutation in KCNE3 identified in a proband with early-onset lone AF possibly leading to gain-of-function of several cardiac currents. We suggest abnormalities in the KCNE3 gene as a potential genetic risk factor for initiation and/or maintenance of AF.

Introduction
Atrial fibrillation (AF) is the most frequent cardiac arrhythmia. The incidence of the disease increases with age, and currently it affects more than 6% of the Western population older than 65 years. It is correlated with increased morbidity and mortality, and as AF increases the risk for thromboembolic events it is a potentially serious disease which can lead to stroke or even heart failure [3]. AF is typically an acquired arrhythmia disease presenting primarily in patients with preexisting cardiovascular disease (including hypertension, ischemic heart disease, valvular disease). In the development of AF in these patients electrical and structural remodeling seems to play a role [4]. However, in rare cases familial occurrence of AF has been described [5] and recent studies have supported that heritable factors may contribute [6, 7]. There is consensus agreement that a
major cause of AF is re-entrance of multiple wavelets in the atria leading to a fast and chaotic electrical activity [8]. Due to a conduction delay of the cardiac impulse at the AV node the fast activity in the atria leads to a random activation of the ventricles. Patients diagnosed with AF will therefore often present with a fast and irregular pulse.

The current conceptual model for AF proposes a shortening of the cardiac action potential refractory period as substrate for the re-entrant wavelets in the atria [9]. As the refractory period is primarily dictated by voltage-gated potassium (Kv) channels underlying the action potential repolarization it is not surprising that dysfunction of exactly these channels has been linked to AF. Specifically, all except one identified mutations related to AF have a feature in common: they induce gain-of-function of cardiac potassium (K+) channel subunits [10-14]. Additionally, a common polymorphism in the gene encoding the cardiac Na,1.5 sodium channel has been correlated with AF [15, 16]. These findings support the model of a shortened refractory period as substrate for AF as the mutations identified are predicted to decrease action potential duration.

In this study we identified a genetic defect in a 46-year old male patient presented with lone AF. The proband carries a missense mutation in the KCNE3 gene encoding a Kv channel accessory subunit. KCNE3 is a single transmembrane protein, and it affects currents generated by a number of Kv channels that contribute to the cellular repolarization in the heart; the most prominent of which are the Ito, Ik, Ik1s and Ikur currents. We investigated the molecular effect of the mutation by performing electrophysiological experiments on heterologously expressed channel proteins underlying these currents in Xenopus laevis oocytes. We found that the mutation identified induces gain-of-function of Kv4.3/KCNE3 and Kv11.1/KCNE3 channel complexes. Based on these findings we suggest abnormalities in the KCNE3 gene as a potential risk factor for AF.

Materials and Methods

Patient presentation

The investigation conforms to the principles outlined in the Declaration of Helsinki. The collection of clinical data and analysis of samples was approved by the Ethics committees of Copenhagen and Frederiksberg counties (KF01-147/02). Patients initially participated in the Copenhagen SAFIR investigation [17] and were included consecutively from the Department of Cardiology, Copenhagen University Hospital Hvidovre, Denmark, from April 1999 to August 2001. The basic inclusion criterion was ECG-documented AF followed by restored sinus rhythm (SR) after DC cardioversion. Three years later 158 of the 220 invited survivors - 50 women and 108 men - attended a follow-up visit with recording of an ECG, an evaluation of cardiac arrhythmia during 24 hours ECG (Holter) recording, registration of disease history and demographic data, a signal averaged ECG, an echocardiogram, and blood samples. The study design was cross-sectional based on a follow-up study of a population characterized by earlier ECG-documented AF followed by SR at the original time of inclusion.

Mutation screening

Genomic DNA was isolated from blood samples from the 158 AF patients and from 96 ethnically matched controls using QIAamp DNA Blood Mini Kit (QIAGEN, Germany). The coding regions of genes investigated were amplified by PCR using primers designed to obtain fragments of appropriate size. Single strand conformation polymorphism (SSCP) of the fragments was performed using GeneGel Excel 12.5/24 kits (Amersham Biosciences AB, Sweden). Aberrant conformers identified in the SSCP were sequenced on a 3100-Avant Genetic analyzer (Applied Biosystems, USA) using Big Dye chemistry.

Molecular biology

The mutation V17M (ATG for GTG) was introduced into human KCNE3 cDNA (Genbank Acc. No. NM_005472) by site-directed PCR mutagenesis using standard overlap PCR techniques and subsequently cloned into the pGEM-HE vector and sequenced using an ABI 377 DNA sequencer (Perkin Elmer/Applied Biosystems, Foster City, CA, USA). cRNA was synthesized from WT and mutant KCNE3 in pGEM-HE, and the human cDNAs for Kv1.5 (in pXOOM), Kv4.3 (in pXOOM), Kv7.1 (in pXOOM), Kv7.4 (in pXOOM), and Kv11.1 (in pXOOM) (Genbank Acc. No. NM_002234, NM_172198, NM_000219, AF105202, and NM_000238, respectively) using the Ambion T7 m-Message Machine kit according to the manufacturer’s instructions (Ambion, USA). cRNA was extracted and dissolved using the MegaClear kit (Ambion, USA). cRNA quality was examined by agarose gel electrophoresis, quantified by UV spectroscopy and stored at -80°C until injection.

Oocyte isolation and injection

Isolation, maintenance and cRNA injection of Xenopus laevis oocytes was performed as previously described [18]. Currents were recorded from oocytes injected with Kv1.5, Kv7.1, Kv7.4, Kv4.3 or Kv11.1 in the absence as well as in the presence of accessory subunits KCNE3 or KCNE3-V17M. In co-expression experiments of α- and β-subunits oocytes were co-injected in a 1:1 molar ratio of channel- and accessory subunits. For paired comparisons only oocytes isolated from the same frog were used.

Two-electrode voltage clamp recordings

Currents were recorded at room temperature 48-96 hours after injection using a two-electrode voltage-clamp amplifier (CA-1B, Dagan, Minneapolis, MN) and PULSE acquisition software (HEKA, Lambrecht, Germany). Oocytes were immersed...
in Kulori medium (90 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, pH 7.4) and subject to a constant flow of Kulori medium during recordings. Electrodes were pulled from borosilicate glass capillaries on a horizontal patch electrode puller and had a tip resistance between 0.3 and 2.0 MΩ when filled with 2 M KCl.

**Data analysis**

All data was analyzed using IGOR software and values shown are mean ± SEM. The number of independent experiments is indicated by n. The voltage dependence of activation was determined by two-state Boltzmann distribution fits to the amplitude of peak tail currents versus test potential. That is, data was fit to functions of the form \( I(V) = \frac{1}{1+\exp\left((V-V_{1/2})/a\right)} \), where \( V_{1/2} \) is the potential for half-maximal activation and \( a \) is the slope factor. Comparison of the biophysical properties for channels co-expressed with either wild type or mutant KCNE3 subunits were performed using an unpaired \( t \)-test. Data was considered significant at \( P<0.05 \).

**Results**

**Clinical analysis and mutation screening**

A 46 year old male patient participating in the SAFIR study presented with lone-AF. The ECG during AF showed a well-regulated ventricular rate (Fig. 1D). The patient received a total of 9 successful cardioversions for AF. He never experienced syncope or near-syncope or other cardiac symptoms. His ECG was normal without signs of hypertrophy or bundle branch block and his echocardiography presented normal as well. The patient was screened for the candidate genes associated with AF (\( KCNQ1, KCNH2, KCNE2, KCNJ2 \)) as well as in five additional genes encoding cardiac K⁺ channel subunits (\( KCNE1, KCNE3-5 \) and \( KCNJ5 \)). We found aberrant conformers on the SSCP analysis for \( KCNE3 \) (Fig. 1A). DNA sequencing revealed the patient heterozygous for a G to A substitution in \( KCNE3 \) nucleotide 49 (Fig. 1B) causing a valine to methionine change in codon 17 (V17M) located in the extracellular N-terminal tail (Fig. 1C) of the KCNE3 protein. The V17 residue is conserved across species suggesting that it either represents a \textit{de novo} mutation or a rare variation. Sequence analysis using common prediction servers (http://www.cbs.dtu.dk/services/) did not indicate disruption of prominent protein functions. The substitution was absent in 96 normal control samples as well as in the remaining 157 AF patients investigated and it was not registered as a Caucasian variant in PharmGKB (www.pharmgkb.com) or in the SNP database of National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). There was no history of AF, sudden cardiac death or sudden infant death in the family of the proband. However, the family of the proband is small: both parents are dead and there are no children. The proband has one sister, who also carries the mutation, but who was asymptomatic at the time she was genotyped (age 48).
Characterization of Kv4.3/KCNE3-V17M channels

We sought to investigate if the mutation in the accessory potassium channel subunit KCNE3 could give rise to the AF phenotype observed in the proband. Therefore, KCNE3-WT or KCNE3-V17M subunits were transiently expressed in the presence of various Kv channels reported to interact with KCNE3 in Xenopus laevis oocytes [18, 19]. The effect of the KCNE3 mutation was assessed by two-electrode voltage-clamp recordings.

We first analyzed the effect of the KCNE3 mutation on Kv4.3 channels. Kv4.3 is a potential molecular substrate for AF as decrease in Kv4.3 mRNA levels in AF patients has been identified [20]. Kv4.3 is believed to be the major subunit underlying the transient outward current, I_{to}, responsible for early repolarization in human atrial myocytes [21]. Recently, it has been shown to interact with KCNE3 in Xenopus laevis oocytes as well as in mammalian cells [18]. Fig. 2A shows representative current traces for Kv4.3 alone and for Kv4.3 co-expressed with KCNE3 or KCNE3-V17M β-subunits. Currents were elicited by the depicted step protocol. Expression of Kv4.3 gave rise to fast activating and inactivating K^+ currents (left panel). Co-expression of KCNE3 subunits led to a drastic inhibition of Kv4.3 currents (middle). KCNE3 almost completely inhibited the transient outward current carried by Kv4.3 channels (~96% inhibition at +60 mV). As evidenced from the current traces (right panel) and as quantified in Fig. 2B the V17M mutation diminished the inhibitory effect of KCNE3 on Kv4.3 generated currents. In the presence of the V17M mutation KCNE3 only induced an inhibition of the transient outward current measured at +60 mV of approximately 65%. The channel complex formed by Kv4.3 and the mutant KCNE3 subunit conducted more current at all potentials tested. In addition to affecting the current amplitude the mutation also interfered with the kinetics of the channel complex (Fig. 2C). The presence of KCNE3 caused a 24 mV shift of the steady-state inactivation of Kv4.3 to more negative potentials (Kv4.3: V_{1/2}=-56±1 mV, a=7±1, n=20 versus Kv4.3/KCNE3: V_{1/2}=-80±1 mV, a=11±1, n=24) whereas KCNE3-V17M induced a significantly smaller left-shift of the inactivation curve of only 14 mV (Kv4.3/KCNE3-V17M: V_{1/2}=-67±1 mV, a=11±1, n=25).

To resemble the state of the patient we further tested the heterozygote state co-expressing Kv4.3 with KCNE3 as well as KCNE3-V17M. The currents generated by the heterozygous channels are identical to the currents carried by Kv4.3/KCNE3-V17M channels (Fig. 2B-C, V_{1/2}=-70±1 mV, a=10±1, n=10). We speculate that the mutation exerts a dominant effect.

Fig. 2. Effect of V17M mutation on Kv4.3/KCNE3 generated currents. (A) Kv4.3 (left), Kv4.3/KCNE3 (middle) and Kv4.3/KCNE3-V17M (right) channels were expressed in Xenopus laevis oocytes and currents were elicited in 10 mV increments to test potentials ranging from -110 to +30 mV from a holding potential of -80 mV. Tail currents were measured at +40 mV. (B) I-V relationship for Kv4.3 (n=20), Kv4.3/KCNE3 (n=24), Kv4.3/KCNE3-V17M (n=25) and Kv4.3/KCNE3/KCNE3-V17M (n=10) channels measured as the transient outward current amplitude upon stimulation. (C) Steady-state inactivation curves for Kv4.3, Kv4.3/KCNE3, Kv4.3/KCNE3-V17M and Kv4.3/KCNE3/KCNE3-V17M channels. Transient outward currents upon depolarization to +40 mV were measured and two-state Boltzmann distributions were fit to the normalized data as function of the pre-pulse potential.
Fig. 3. The V17M mutation alters Kv11.1/KCNE3 generated currents. (A) Representative current traces recorded from *Xenopus laevis* oocytes injected with Kv11.1 (left), Kv11.1/KCNE3 (middle) or Kv11.1/KCNE3-V17M (right) cRNA. Currents were elicited in 20 mV steps ranging from -120 mV to +40 mV and tail currents were measured at -120 mV. (B) I-V relationship for Kv11.1, Kv11.1/KCNE3 or Kv11.1/KCNE3-V17M channels measured at the end of the test pulse. (C) Histogram summarizing time constants for deactivation obtained by single exponential fits to tail currents measured subsequent to a +40 mV stimulus.

**Characterization of Kv11.1/KCNE3-V17M channels**

We next investigated the effect of the KCNE3 mutation on currents generated by Kv11.1 in complex with KCNE3. Kv11.1 is thought to underlie the rapidly activating component of the delayed rectifier current, $I_{Kr}$, in human cardiomyocytes, which is active during the action potential plateau phase and the final repolarization [22]. Previous studies have shown that Kv11.1 currents are inhibited by KCNE3 in heterologous expression systems [19] and that KCNE3 can significantly reduce $I_{Kr}$ in human cardiomyocytes [23]. In Fig. 3A representative current traces recorded from *Xenopus* oocytes expressing Kv11.1, Kv11.1/KCNE3 or Kv11.1/KCNE3-V17M channels are shown. Channels were activated by 20 mV voltage increments ranging from -120 mV to +40 mV. Depolarization of the membrane potential evoked channel activation followed by rapid channel inactivation. Upon membrane repolarization to -120 mV inactivation was released and channels slowly deactivated. The strong inactivation characterizing these channels is further evidenced in the bell-shaped I-V relationship outlined in Fig. 3B, which shows the current amplitude measured at the end of the test pulse as function of the membrane potential. As evidenced in Fig. 3A-B Kv11.1 channels conducted less current in the presence of the KCNE3 subunit. However, the inhibitory effect of the KCNE3 subunit was completely abolished by the V17M mutation. Further, KCNE3 affected the deactivation of Kv11.1 channels as it accelerated deactivation (Fig. 3C, $\tau_{Kv11.1} = 127\pm6$ ms versus $\tau_{Kv11.1/KCNE3} = 67\pm5$ ms). This effect of KCNE3 on deactivation was abolished by the V17M mutation ($\tau_{Kv11.1/KCNE3-V17M} = 131\pm5$ ms). That is, channel complexes containing mutant KCNE3 subunits conduct more current than wild type channel complexes do.

We did not observe any differences in the activation kinetics between Kv11.1 ($V_{1/2}=-29\pm1$ mV, $a=8\pm1$, $n=20$), Kv11.1/KCNE3 ($V_{1/2}=-28\pm1$ mV, $a=8\pm1$, $n=22$) or Kv11.1/KCNE3-V17M ($V_{1/2}=-31\pm3$ mV, $a=7\pm2$, $n=20$) as found by two-state Boltzmann fits to normalized peak tail currents.

**Characterization of KCNE3-V17M in complex with Kv1.5, Kv7.1 and Kv7.4 channels**

Additionally we investigated the effect of the mutation on the $\alpha$–subunits underlying the $I_{Ks}$ and $I_{Kur}$ currents. $I_{Ks}$ is the slow component of the delayed rectifier current and it is active during the action potential plateau phase and the final repolarization whereas the ultra-rapid $K^+$ current, $I_{Kur}$ plays a role during early- and plateau phase repolarization. Kv7.1 is the main carrier of $I_{Ks}$ and Kv1.5 is the main carrier of $I_{Kur}$ [24, 25]. Hence, we investigated the effect of KCNE3-V17M on Kv7.1/KCNE3 and Kv1.5/KCNE3 channel complexes.

Figure 4A shows representative current traces elicited by the depicted step protocol after injection of Kv7.1 cRNA either by itself or in conjunction with wild type or mutant KCNE3 cRNA. Expression of Kv7.1 channels.

KCNE3 and AF
gave rise to small, fast activating voltage-dependent K+ currents (left). Co-expression of KCNE3 changed the biophysical properties of Kv7.1 channels as previously described (upper right) [19, 26]. Channel complexes comprised of Kv7.1 and KCNE3-V17M conducted currents indistinguishable from those generated by the co-assembly of Kv7.1 and KCNE3 subunits (lower right). Figure 4B depicts current traces after injection of Kv1.5 cRNA. Application of a standard voltage-step protocol (upper left) elicited fast activating, slowly inactivating currents. Neither co-expression of wild type nor mutant KCNE3 subunits affected Kv1.5 generated currents.

However, the V17M mutation did not induce any changes to currents generated by this channel complex (lower right).

Discussion

Atrial fibrillation is a progressive cardiac arrhythmia disorder characterized by chaotic electrical activity in the atria predisposing for stroke and heart failure. Structural and electrical remodeling have been shown to play a major role in the arrhythmogenesis [28]. Electrical remodeling involves alterations in the electrophysiologic milieu of myocardium primarily due to alterations in function of ion channels. In AF, several currents are down-regulated including the calcium and potassium currents $I_{Ca,L}$, $I_{Kto}$, and $I_{kur}$ (for review see [29]). However, it is increasingly recognized that heritable factors possibly contribute to the disorder; hence, there is growing interest in identifying genetic defects conferring AF susceptibility. Such genetic...
defects are particularly likely to be identified in patients presented with early-onset lone AF. These patients have apparently normal hearts and no traditional risk factors and thus allow for investigation of genetic defects as cause of AF with a minimum of confounding variables. Here we report a KCNE3 missense mutation identified in a proband with lone AF. As the substitution was not found in 96 unrelated healthy controls or in 157 unrelated AF patients and is conserved across species it is likely that the substitution represents a de novo mutation or a rare variation. The mutation resides in the N-terminus of the protein to which no clear function has been allocated so far. The mutation disrupts the inhibitory effect of KCNE3 on Kv4.3 and Kv11.1 currents, and thus provides a novel molecular substrate for AF. Previous studies have linked AF to impaired Kv channel function. Missense mutations in the cardiac potassium channel subunits KCNQ1, KCNE2, KCNJ2, KCNH2 induce atrial fibrillation via shortening of the action potential duration and atrial effective refractory period conferred through gain-of-function mutations (for review, see [30]). These findings correlate well with the hypothesis that action potential refractory period shortening is a substrate for re-entrant wavelets in the atria and thereby a substrate for AF as an increase in Kv channel function leads to a faster action potential repolarization. However, for KCNA5 a loss-of-function mutation has been reported producing an atrial action potential prolongation pointing to a mechanistic heterogeneity for the development of AF [13].

Various outward K+ currents contribute to the repolarization in the heart; the most prominent of which are the I_to, I_k1, I_k2, and I_kur currents conferred by Kv4.3, Kv11.1, Kv7.1 and Kv1.5 α-subunits respectively. We addressed the effect of the mutation V17M in KCNE3 on those currents. KCNE3 has been detected in the heart on mRNA as well as on protein level [19, 31, 32], but its functional role in cardiac electrical activity remains to be elucidated. However, as injection of KCNE3 adenovirus into left ventricle of guinea pig heart has been shown to reduce action potential duration by 66% and QTc by 10% KCNE3 appears to play a significant role in cardiac electrical activity [23].

In this study we show that the V17M mutation in KCNE3 diminishes the inhibitory effect of KCNE3 on Kv4.3 channels; the mutation thus induces a gain-of-function for the Kv4.3/KCNE3 channel complex. Assuming that KCNE3 interacts with Kv4.3 in cardiac tissue in vivo the reduced inhibitory effect of the mutation would lead to an increase in I_to. Unexpectedly, a previous study found I_to current density in AF patients to be decreased compared to persons in sinus rhythm [20]. However, since I_to is one of the major outward currents in human atria, the current should be largely inhibited when interacting with KCNE3 in normal cells. Further studies have to be undertaken to clarify if KCNE3 plays an important role in the native myocyte in normal or diseased conditions. Our investigations further showed that the V17M mutation abolished the inhibitory effect of KCNE3 on Kv11.1 generated currents. As Kv11.1 is proposed to underlie the rapidly activating component of the cardiac delayed rectifier current, I_Ks, assuming that Kv11.1 and KCNE3 interact in the heart in vivo would thus result in an increased I_Ks current for KCNE3-V17M carriers. As a Kv11.1 gain-of-function mutation inducing AF has been described [10], the gain-of-function effect we observe for V17M on Kv11.1/KCNE3 channel complexes may be a molecular explanation for AF in this patient.

Taken together we found the V17M mutation to decrease the inhibitory effect of KCNE3 on two of the major K+ channels underlying cardiac repolarization. This effect is not due to the mutation rendering the KCNE3 protein non-functional per se as the mutant subunit was found to confer effects similar to wild type subunit upon co-expression with Kv7.1 and Kv7.4 channel proteins. Assuming that KCNE3 interacts with Kv4.3 and Kv11.1 in human atria our findings suggest that V17M mutation carriers would be subject to acceleration of action potential repolarization and thus a shortened effective atrial refractory period. Performing RT-PCR experiments we were able to detect transcripts of KCNE3, Kv4.3 and Kv11.1 in both left and right compartments of the atria and ventricles (data not shown).

The observation that the sister of the proband also carries the KCNE3 V17M mutation but does not present with AF could imply that the KCNE3 V17M mutation is either a predisposing factor for AF, or that environmental factors might also be involved in the onset of AF, or it might simply reflect a low penetrance.

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