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A Novel Channelopathy in Pulmonary Arterial Hypertension

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Abstract

BACKGROUND—Pulmonary arterial hypertension is a devastating disease with high mortality. Familial cases of pulmonary arterial hypertension are usually characterized by autosomal dominant transmission with reduced penetrance, and some familial cases have unknown genetic causes.

METHODS—We studied a family in which multiple members had pulmonary arterial hypertension without identifiable mutations in any of the genes known to be associated with the disease, including *BMPT2*, *ALK1*, *ENG*, *SMAD9*, and *CAV1*. Three family members were studied with whole-exome sequencing. Additional patients with familial or idiopathic pulmonary arterial hypertension were screened for the mutations in the gene that was identified on whole-exome sequencing. All variants were expressed in COS-7 cells, and channel function was studied by means of patch-clamp analysis.

RESULTS—We identified a novel heterozygous missense variant c.608 G → A (G203D) in *KCNK3* (the gene encoding potassium channel subfamily K, member 3) as a disease-causing candidate gene in the family. Five additional heterozygous missense variants in *KCNK3* were independently identified in 92 unrelated patients with familial pulmonary arterial hypertension and 230 patients with idiopathic pulmonary arterial hypertension. We used in silico bioinformatic tools to predict that all six novel variants would be damaging. Electrophysiological studies of the channel indicated that all these missense mutations resulted in loss of function, and the reduction in the potassium-channel current was remedied by the application of the phospholipase inhibitor ONO-RS-082.

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CONCLUSIONS—Our study identified the association of a novel gene, *KCNK3*, with familial and idiopathic pulmonary arterial hypertension. Mutations in this gene produced reduced potassium-channel current, which was successfully remedied by pharmacologic manipulation. (Funded by the National Institutes of Health.)

Pulmonary Arterial Hypertension Is a rare disease that is characterized by increased pulmonary-artery pressure in the absence of common causes of pulmonary hypertension, such as chronic heart, lung, or thromboembolic disease.¹ Before the advent of novel therapies, patients with idiopathic or familial pulmonary arterial hypertension had an estimated median survival of 2.8 years, with 1-year, 3-year, and 5-year survival rates of 68%, 48%, and 34%, respectively.² However, despite progress in treatment, pulmonary arterial hypertension remains a progressive, fatal disease. The clinical presentation can be nonspecific, and patients often receive a diagnosis late in their clinical course.

The cause of pulmonary arterial hypertension is heterogeneous, and some cases are familial. Molecular genetic studies have shown that mutations in the gene encoding bone morphogenetic protein receptor type II (*BMPR2*) are present in approximately 70% of patients with familial pulmonary arterial hypertension, as well as in 10 to 25% of those with idiopathic pulmonary arterial hypertension.^{3–5} Pulmonary arterial hypertension may also occur in patients carrying mutations in the gene encoding activin receptor–like kinase 1 (*ALK1*) and more rarely in patients carrying mutations in the gene encoding endoglin (*ENG*); mutations in both genes are known to cause hereditary hemorrhagic telangiectasia.^{3,6–9} In rare cases, mutations in the gene encoding mothers against decapentaplegic homologue 9 (*SMAD9*) have been identified in patients with idiopathic pulmonary arterial hypertension.^{10,11} We previously identified novel mutations in the gene encoding caveolin 1 (*CAVI*) in patients with either familial or idiopathic pulmonary arterial hypertension.¹² In approximately 25% of patients with familial pulmonary arterial hypertension, there is no identifiable genetic cause.

In this study, we used whole-exome sequencing to identify a novel cause of pulmonary arterial hypertension in a family with this disorder, replicated our findings in patients with either familial or idiopathic pulmonary arterial hypertension, and characterized the loss of channel function for each mutation.

Methods

STUDY PARTICIPANTS AND GENETIC STUDIES

We studied a family in which pulmonary arterial hypertension had been diagnosed in five members (two living and three deceased at the time of the analysis) (Fig. 1A). The diagnosis of pulmonary arterial hypertension was confirmed by means of medical-record review and rightheart catheterization. Written informed consent for genetic studies was obtained from all the participants. The study was funded by the National Institutes of Health, and the protocol was approved by the appropriate human-subjects committees. Details of the methods are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org. DNA from the family members had been sequenced previously to establish that they did not carry *BMPR2*, *ALK1*, *ENG*, *SMAD9*, or *CAVI* mutations.

We used whole-exome sequencing to compare the three affected family members, assuming an autosomal dominant mode of inheritance, and variants were filtered on the basis of allele frequency in controls and predicted pathogenicity. A novel variant was identified in *KCNK3* (the gene encoding potassium channel subfamily K, member 3), and Sanger sequencing of *KCNK3* was performed on samples obtained from all available members of the study family to assess for cosegregation with disease. To identify additional mutations and mutation

carriers, DNA samples from 82 unrelated patients with familial pulmonary arterial hypertension and 230 patients with idiopathic pulmonary arterial hypertension were sequenced, and whole-exome sequencing data from 10 additional patients with familial pulmonary arterial hypertension were reviewed, to replicate the findings in the initial family and determine the frequency of mutations in *KCNK3* in patients with familial and idiopathic pulmonary arterial hypertension. For patients with familial pulmonary arterial hypertension who were found to have *KCNK3* mutations, other available family members were tested to evaluate segregation within the family.

LUNG-TISSUE SAMPLING

Lung tissue was obtained from explanted lungs of two patients with idiopathic pulmonary arterial hypertension. The specimens were fixed in 10% formalin, processed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, CD31, alpha-smooth-muscle actin, or von Willebrand factor, along with Verhoeff–van Gieson elastic stain.

EXPRESSION AND FUNCTIONAL ANALYSIS OF HUMAN KCNK3 CHANNEL

We performed functional analysis of the human KCNK3 (hKCNK3) channel to evaluate the genetic variants that had been identified. Mutations were engineered into hKCNK3 complementary DNA (cDNA) and expressed with the use of transient transfection in COS-7 cells. Whole-cell patch-clamp procedures were used to measure expressed currents and their response to pH and pharmacologic agents. Detailed methods for the molecular biologic and electrophysiological studies are provided in the Supplementary Appendix.

Results

WHOLE-EXOME SEQUENCING

The average depth of sequence coverage of the whole-exome sequencing data was 78.7×, with 87.5% of the target region for exome capture having coverage of more than 20×. We removed variants that had an allele frequency of more than 1% in established databases, including dbSNP, the 1000 Genomes Project, and the National Heart, Lung, and Blood Institute Exome Variant Server. This left 4719 rare or novel variants that were present in at least one of the three affected family members. We filtered these variants to identify heterozygous variants shared by the three affected family members and were left with 377 novel single-nucleotide variants (SNVs) and 6 insertions or deletions (indels). Because the pedigree suggested an autosomal dominant mode of inheritance, homozygous variants were excluded.

Variants were then filtered for predicted pathogenic effects with the use of a series of in silico bioinformatic tools (see the Supplementary Appendix). A total of 19 SNVs and 5 indels were predicted to be deleterious. Of these, a novel missense variant, c.608 G → A (G203D), in *KCNK3* was identified as the strongest candidate because *KCNK3* is reported to be important in the regulation of pulmonary vascular tone in humans.¹³ The function of this channel is to conduct potassium current, maintain resting membrane potential, and regulate the vascular tone of the pulmonary artery.^{14–16} On the basis of homologic modeling of hKCNK3, amino acid G203 is located in the highly conserved second pore region of the protein, which is critical for the gating function of the potassium channel. Examination of dbSNP indicated that there are no common missense variants (allele frequency, >1%) in *KCNK3*.

CONFIRMATION OF THE MUTATION

Segregation analysis of the c.608 G → A variant in *KCNK3* was performed on all available members of the index family (Fig. 1A). Affected Family Member II-1 was found to carry the

variant, but her unaffected brother-in-law (Family Member II-3) was not. Her affected sister (Family Member II-2) was deceased at the time of our study and had no DNA available for testing, but it was assumed that she had also carried the variant, since four of her children (Family Members III-1, III-2, III-4, and III-5) were all carriers. Although Family Member III-5 was initially unaffected at the time of recruitment, he subsequently received a diagnosis of pulmonary arterial hypertension. Family Member III-2 remains unaffected. The c.608 G → A *KCNK3* variant was not present in DNA samples from 100 ethnically matched, unrelated, unaffected white controls.

CASE SERIES OF PATIENTS

We also used whole-exome sequencing to study samples obtained from 10 additional probands from families with familial pulmonary arterial hypertension. Two novel heterozygous *KCNK3* variants, G97R and V221L, were identified in two of these families. These variants were confirmed on Sanger sequencing and tested in available family members and were found to segregate with disease (Fig. 1B and 1C). We screened an additional 82 unrelated probands from families with familial pulmonary arterial hypertension and 230 patients with idiopathic pulmonary arterial hypertension for mutations in *KCNK3*. Among the latter group of patients, we identified three novel heterozygous amino acid substitutions: T8K, E182K, and Y192C. The five additional variants were all predicted to be damaging. *KCNK3* mutations were found in 3 of 230 participants (1.3%) in the cohort of patients with idiopathic pulmonary arterial hypertension and in 3 of 93 participants (3.2%) in the cohort of probands with familial disease.

CLINICAL PHENOTYPES OF MUTATION CARRIERS

In the three families with familial pulmonary arterial hypertension, two of nine family members who inherited a *KCNK3* mutation did not have evidence of disease, suggesting incomplete penetrance. Among all the patients with clinical evidence of pulmonary arterial hypertension (familial or idiopathic), the age at diagnosis ranged from 8 to 44 years (Table 1). Both male and female patients were affected. No patient had a response to acute vasodilator challenge. Three patients ultimately required lung transplantation. The histopathological analysis for one patient who underwent lung transplantation is shown in Figure 1 in the Supplementary Appendix. Findings included hypertrophy of the media of muscular pulmonary arteries and progressive, generalized arterial dilatation with formation of complex plexiform lesions.

FUNCTIONAL STUDIES OF HUMAN *KCNK3* CHANNEL

KCNK3 encodes a pH-sensitive potassium channel in the two-pore domain superfamily.¹⁷ The primary role of *KCNK3* channels is to control the resting membrane potential in many cell types, including human pulmonary-artery smoothmuscle cells,¹⁷ and to contribute to arterial relaxation through the action of smooth-muscle cells. *KCNK3* channels lack voltage dependence. Alignment of the *KCNK3* channel with other two-pore domain potassium channels reveals that most of the mutations found in this study occurred at conserved residues that were likely to be critical for function (Fig. 2).

To investigate the consequences of *KCNK3* variants, we studied the effect of the six mutations that were discovered in our genetic studies of the h*KCNK3* channel. Nonmutant channels and all mutant channels were tested for pH sensitivity to confirm their identity as h*KCNK3* channels. The pH dependence of nonmutant h*KCNK3* channels is shown in Figure 3A. All mutants that were tested resulted in loss of function at physiologic pH (7.4) when expressed alone, a condition that simulates homozygous expression in humans (Fig. 3B and 3C). However, because two-pore-domain potassium channels assemble as dimers¹⁶ and patients carrying mutations are heterozygotes, we also coexpressed nonmutant and mutant

hKCNK3 channel cDNA to simulate expression in heterozygous patients. For these experiments, we chose three mutations located in distinct regions of the channel and found that the mutations studied (Y192C, G203D, and V221L) reduced current density when coexpressed with nonmutant channels, as compared with expression of nonmutant hKCNK3 channels alone (Fig. 3D).

A number of compounds, including the phospholipase A2 inhibitor ONO-RS-082, have been shown to activate nonmutant hKCNK3 channels.¹⁸ Thus, we sought to determine whether this drug was capable of rescuing channel activity in the hKCNK3 mutant channels in this study. We found recovery of current for some, but not all, disease-associated mutants. Shown in Figure 4 are examples of current recordings with ONO-RS-082 (10 μ M) and without ONO-RS-082 for nonmutant and mutant hKCNK3 channels (Fig. 4A) as well as current density (at +60 mV) before, during, and after application of the drug (Fig. 4B). Steady-state effects of the drug are summarized in Figure 4C. The results indicate a robust increase in nonmutant hKCNK3 current after application of ONO-RS-082 and an increase in current density to levels similar to those seen in the nonmutant channel for two mutant channels (T8K and E182K) but not a third mutant channel (G203D).

Discussion

We used whole-exome sequencing to identify the association of a novel gene, *KCNK3*, with pulmonary arterial hypertension in a family that had multiple affected members. We also identified mutations in *KCNK3* in other families with familial pulmonary arterial hypertension and in patients with idiopathic pulmonary arterial hypertension and showed that all such mutations resulted in a loss of ion-channel function. These findings suggest that *KCNK3* is involved in the pathogenesis of pulmonary arterial hypertension.

KCNK3, also called TASK-1, belongs to a family of mammalian potassium channels that is characterized by the presence of four transmembrane domains and two pore domains per subunit.¹⁹ It has been reported that this potassium channel is sensitive to hypoxia and plays a role in the regulation of resting membrane potential and pulmonary vascular tone.^{13-15,20} Ion channels also play a critical role in vascular remodeling, and it has been postulated that *KCNK3* is involved in the regulation of vascular remodeling and abnormal vascular proliferation in patients with pulmonary arterial hypertension by preventing apoptosis.²¹ *KCNK3* knockout mice have a blunted (although not abolished) ventilatory response to hypoxia.²² Quantitative analysis of oxygen-sensing and pulmonary-artery pressures has not, to our knowledge, been reported in these mutant mice. *KCNK3* is expressed in human pulmonary-artery smooth-muscle cells, and knockdown of *KCNK3* has been shown to cause membrane depolarization and reduced potassium current.¹³ Taken together, these results strongly suggest that *KCNK3* is important in the regulation of pulmonary vascular tone.

As indicated by our electrophysiological studies, the variants that were identified in this study are all loss-of-function mutations. Because *KCNK3* channels are not voltage-dependent and are open at negative potentials, these mutations probably cause depolarization of the resting membrane potential, which could lead to pulmonary-artery vasoconstriction.²³ The molecular mechanisms for loss of function probably vary according to the location of the mutation in the channel. One mutation, T8K, is in the N-terminal, a part of the channel that is important to membrane transport out of the endoplasmic reticulum through interaction with 14-3-3 proteins.²⁴ Four of the mutations fall in the pore domains in *KCNK3* that are critical for the pH sensitivity and potassium selectivity of this potassium-channel family.^{25,26} Two of the mutations, G97R and G203D, are in the pore-domain GXG triplet selectivity filters (in which X is any amino acid) of *KCNK3* (Fig. 2A) and may have

their deleterious effects as a result of alterations in potassium selectivity. The last mutation falls in one of the transmembrane domains that have been implicated by structural models as important for dimerization.^{16,26} Each mutation we identified (possibly excepting T8K) falls in a highly conserved region of *KCNK3* (Fig. 2B), indicating that these residues are important for the normal biophysical properties of the KCNK3 channel. There are parallels to our findings in studies of voltage-gated potassium (Kv) channels in human pulmonary-artery smooth-muscle cells in that down-regulation of Kv channels has been implicated in altered contraction and proliferation in smooth-muscle cells in patients with primary pulmonary hypertension.²⁷

Although *KCNK3* is expressed in multiple tissues, including heart, brain, and pancreas, the mutations in *KCNK3* that we identified were associated only with pulmonary arterial hypertension in the patients we studied. This finding may be due to redundancy within the two-pore-domain potassium channels. *KCNK9* is expressed in the brain,¹³ *KCNK5* and *KCNK6* are abundant in the pancreas,²⁸ and *KCNK1* is expressed in the heart.²⁹ This redundancy may explain why the phenotype of mutations in *KCNK3* is specific to pulmonary hypertension.

We found that the function of channels incorporating mutant KCNK3 can be rescued (to a variable degree, depending on the mutation) with the use of the phospholipase inhibitor ONO-RS-082. Other pharmacologic interventions may activate KCNK3 as well. In human pulmonary-artery smooth-muscle cells, KCNK3 can be activated by treprostinil (a stable prostacyclin analogue) through cyclic AMP (cAMP)-dependent phosphorylation of the channel induced by protein kinase A (PKA).^{30,31} Application of the cAMP analogue 8-bromo-cAMP, an endogenous PKA activator, also results in KCNK3 activation.¹³ In addition, KCNK3 has been shown to mediate vasoconstriction induced by endothelin 1,³² and application of the specific Rho kinase inhibitor Y-27632 can attenuate endothelin-1-induced KCNK3 inhibition.³³ Thus, our study suggests a potential novel mechanism for therapeutic intervention by pharmacologically increasing currents through KCNK3 in patients with pulmonary arterial hypertension.

It is also possible that in patients with pulmonary arterial hypertension, variation in KCNK3 function may be a more broadly applicable risk factor (or a secondary disease modifier) that is not caused by mutations in *KCNK3*. There is precedent for this concept, since *BMPR2* expression is reduced in the lungs of patients with idiopathic pulmonary arterial hypertension who do not have *BMPR2* mutations.³⁴ In addition, previous studies of Kv channels support the concept that the expression or function of Kv channels is altered in patients with idiopathic pulmonary arterial hypertension, and dysfunctional Kv-channel activity may contribute to the development or persistence of pulmonary arterial hypertension.³⁵ In a study of mice with wild-type Kv channels, therapeutic Kv-channel activation was useful in the treatment of established pulmonary arterial hypertension in the absence of known genetic variations in Kv channels.³⁶ Thus, the therapeutic targeting of KCNK3 may be beneficial for patients with pulmonary arterial hypertension who have increased vascular tone independent of their KCNK3 genetic status.

In our study, two members of families with familial pulmonary arterial hypertension who had inherited *KCNK3* mutations had no evidence of disease. These family members may be examples of incomplete penetrance or of late-onset disease that has not yet developed. Other genetic forms of pulmonary arterial hypertension have incomplete penetrance, and the disease develops at a wide range of ages. Presumably, there are other genetic, environmental, or developmental modifiers that in concert with KCNK3 dysfunction determine whether or when pulmonary arterial hypertension will develop. Identifying

asymptomatic persons who are genetically at risk provides a potential opportunity for early intervention and treatment if an effective therapy is available.

In conclusion, in patients with either familial or idiopathic pulmonary arterial hypertension, we have identified mutations in the potassium channel KCNK3 that represent a mechanistically novel cause of pulmonary arterial hypertension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Pedigrees of Families with Familial Pulmonary Arterial Hypertension
 Segregation of *KCNK3* mutations c.608 G → A (G203D) in the index family (Family 1) (Panel A), c.289G → A (G97R) in Family 2 (Panel B), and c.661G → C (V221L) in Family 3 (Panel C) is indicated. The sequence shown in Panel C is the sequence of the complementary strand. Arrows show the family members whose DNA was analyzed with the use of whole-exome sequencing. Genotypes of family members are shown under each symbol. NM/NM denotes nonmutated homozygote, and NM/M heterozygote with one copy of the *KCNK3* mutation. The current age or the age at death, as well as the age at diagnosis (Dx), where applicable, is provided for each family member. Black squares denote affected

males, black circles affected females, white squares unaffected males, and white circles unaffected females; slashes indicate deceased family members.

Figure 2. Topologic Analysis of the Human KCNK3 (hKCNK3) Channel and Sequence Alignment with Other Members of the KCNK Channel Family

Panel A shows a topologic analysis of the hKCNK3 channel, indicating the positions of the mutations that were identified in this study. Panel B shows the alignment of the amino acid sequences of KCNK3 with three other acid-sensitive members of the KCNK channel family and KCNK1. The positions of the mutations are indicated by the various colors. COOH denotes C-terminal.

Figure 3(facing page). Functional Consequences of hCKNK3 Mutations

Whole-cell patch-clamp procedures were used to measure expressed currents and their response to pH and pharmacologic agents. Panel A shows the representative pH dependence of the current in the nonmutant (NM) hCKNK3 channel. Dashed lines indicate current density at a pH of 7.4. For each point, 4 to 14 cells were studied. The solid curve shows the best fit for the dose-response values. Currents were measured at +60 mV and normalized to current measured at a pH of 10.4. Panel B shows current traces at a pH of 7.4 for the nonmutant hCKNK3 channel and the T8K, G97R, E182K, Y192C, G203D, and V221 mutants. Current density is measured as picoamperes per picofarad (pA/pF). For all current traces, the vertical scale is 10 pA/pF and the horizontal scale is 20 mV. The inset shows the

ramp protocol (i.e., voltage steps or ramps). The vertical dashed lines represent the current at 60 mV. Panel C shows a summary of results illustrated in Panel B, according to mutation. Panel D shows a comparison between the homozygous nonmutant hKCNK3 channel and heterozygous channels incorporating the Y192C, G203D, or V221L mutant at a pH of 7.4. For every point, 7 to 25 cells were studied. In Panels C and D, data are shown as means; T bars indicate standard errors. Asterisks denote $P < 0.05$ for the comparison between the nonmutant hKCNK3 channel and each mutant.

Figure 4(facing page). Pharmacologic Recovery of Mutant hKCNK3 Channels

The phospholipase A2 inhibitor ONO-RS-082 has been shown to activate nonmutant (NM) hKCNK3 channels. Panel A shows the representative recordings before the application of ONO-RS-082 (gray lines) and after the application (black lines) in nonmutant and mutant hKCNK3 channels. For all current traces, the vertical scale is 8 pA/pF. Panel B shows the time course of drug application before (gray squares), during (black circles), and after (gray triangles) application. Arrows indicate the current-density level before drug application. Panel C shows a summary of results of drug effects on nonmutant and mutant hKCNK3 channels. Light blue bars represent the current before drug application; dark blue bars represent the maximal drug response. Data are shown as means; T bars indicate standard

errors. Asterisks indicate $P < 0.05$ for the comparison between the current before drug application and the maximal drug response, as calculated by means of the paired Student's *t*-test.

Table 1
Clinical Characteristics of Seven Patients with Pulmonary Arterial Hypertension with *KCNK3* Mutations*

Characteristic	Family 1 Member 11-2	Family 1 Member III-1	Family 2 Member III-1	Family 3 Member 11-2	Patient 1	Patient 2	Patient 3
Sex	Female	Female	Male	Female	Male	Female	Male
Age at diagnosis (yr)	44	37	19	29	25	38	8
Current age (yr)	Deceased at 46	Deceased at 49	20	Deceased at 40	40	43	20
Lung transplantation	No	No	No	At age 33	At age 29	No	At age 15
<i>KCNK3</i> variant	G203D	G203D	G97R	V221L	E182K	T8K	Y192C
Type of pulmonary arterial hypertension	Familial	Familial	Familial	Familial	Idiopathic	Idiopathic	Idiopathic
Mean pulmonary-artery pressure at diagnosis (mm Hg)	76	62	86	67	101	54	107
Right atrial pressure at diagnosis (mm Hg)	18	7	11	7	16	24	3
Pulmonary capillary wedge pressure at diagnosis (mm Hg)	13	10	13	7	15	6	10
Cardiac index at diagnosis (liters/min/m ²)	2.74	3.16	3.22	2.72	1.73	1.21	2.70
Pulmonary vascular resistance index at diagnosis (dyn-sec-cm ⁻⁵)	1839	1316	1813	1764	3977	3174	2874
NYHA functional class at diagnosis	NA	NA	III	III	III	III	III
Response to acute vasodilator challenge	NA	NA	No	No	No	No	No
Arrhythmias	Partial right bundle-branch block				Atrial flutter	First-degree atrioventricular block; right bundle-branch block	

* Family 1 members 11-2 and III-1, Family 2 member III-1, and Family 3 member 11-2 all received the diagnosis of familial pulmonary arterial hypertension. Patients 1, 2, and 3 received the diagnosis of idiopathic pulmonary arterial hypertension. NA denotes not available, and NYHA New York Heart Association.