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Abstract: Genetic variation is an important determinant of atrial fibrillation (AF) susceptibility. Numerous rare variants in protein-coding sequences of genes have been associated with AF in families and in early-onset cases, and chromosomal loci harbouring common risk variants have been mapped in AF cohorts. Many of these loci are in non-coding regions of the human genome and are thought to contain regulatory sequences that modulate gene expression. Disease genes implicated to date have predominantly encoded cardiac ion channels, with predicted mutation effects on the atrial action potential duration. More recent studies have expanded the spectrum of disease-associated genes to include myocardial structural components and have highlighted an unsuspected role for cardiac transcription factors. These paradigm-shifting discoveries suggest that abnormalities of atrial specification arising during cardiac development might provide a template for AF in later adult life. With the escalating pace of variant discovery, there is an increasing need for mechanistic studies not only to evaluate single variants, but also to determine the collective effects of each person's burden of rare and common genetic variants, co-morbidities and lifestyle factors on the atrial substrate for arrhythmogenesis. Elucidation of an individual's genetic predisposition and modifiable environmental risk factors will facilitate personalised approaches to AF treatment.

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Dr Ann Gregory
Commissioning Editor
Heart Lung Circulation

Re: "**GENETICS OF ATRIAL FIBRILLATION: STATE OF THE ART IN 2017**"

Dear Ann

We would like to thank you for the opportunity to submit the above article for consideration of publication in Heart Lung Circulation. This is an Invited Review for inclusion in the August Special issue: "Atrial Fibrillation".

We are very excited to have two international authors on this manuscript. Dr Patrick Ellinor and Dr Stephen Lubitz, from the Massachusetts General Hospital, Boston, are leading international players in atrial fibrillation genetics, and have kindly agreed to contribute to our review. As discussed by email, they have some "hot" data that we could potentially include, but are awaiting formal lifting of their own publication embargo. If there is a window of time to include this before the August deadline for the Special Issue, that would be great. We will let you know how they are progressing over the next few months.

This paper is not under consideration for publication elsewhere and all authors have read and approved the manuscript. Dr Ellinor has one disclosure but all other authors have no conflicts of interest.

Yours Sincerely,

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GENETICS OF ATRIAL FIBRILLATION: STATE OF THE ART IN 2017

Short title: Genetics of AF

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Abstract

Genetic variation is an important determinant of atrial fibrillation (AF) susceptibility. Numerous rare variants in protein-coding sequences of genes have been associated with AF in families and in early-onset cases, and chromosomal loci harbouring common risk variants have been mapped in AF cohorts. Many of these loci are in non-coding regions of the human genome and are thought to contain regulatory sequences that modulate gene expression. Disease genes implicated to date have predominantly encoded cardiac ion channels, with predicted mutation effects on the atrial action potential duration. More recent studies have expanded the spectrum of disease-associated genes to include myocardial structural components and have highlighted an unsuspected role for cardiac transcription factors. These paradigm-shifting discoveries suggest that abnormalities of atrial specification arising during cardiac development might provide a template for AF in later adult life. With the escalating pace of variant discovery, there is an increasing need for mechanistic studies not only to evaluate single variants, but also to determine the collective effects of each person's burden of rare and common genetic variants, co-morbidities and lifestyle factors on the atrial substrate for arrhythmogenesis. Elucidation of an individual's genetic predisposition and modifiable environmental risk factors will facilitate personalised approaches to AF treatment.

Key words

Genetics, atrial fibrillation, genomic risk score, ion channels, transcription factors.

Introduction

Atrial fibrillation (AF) is a heritable disorder and substantial progress has been made over the past decade in elucidating its genetic underpinnings. Numerous rare variants that putatively cause AF have been identified in families and in sporadic cases, and chromosomal loci have been mapped for common variants that affect AF susceptibility in the general population. These studies have established a key role for ion channel defects in generating a substrate for AF with both gain-of-function and loss-of-function mechanisms demonstrated. More recent data have expanded our perspectives on how ion channel variants promote AF, with evidence for epistatic effects of combinations of variants and gene-environment interactions. Genetic variants in a broad range of non-ion channel genes can also affect AF risk. In particular, the finding of variants within and in the vicinity of genes encoding cardiac transcription factors has opened a new avenue of investigation into how cardiac developmental abnormalities might predispose to AF in later life. Despite these advances, genetic testing of patients with AF has been limited. Current knowledge about the molecular basis of AF and strategies to incorporate genetic information into clinical management will be summarized in this review.

Variant discovery

Rare variants

Much of what we know about genetic causes of AF has been obtained from studies of cohorts of individuals with early-onset (<66 years) lone AF or families in which AF segregates as a Mendelian trait. These patient groups have a high *a priori* likelihood of a genetic aetiology of AF, and there is an expectation that this is primarily due to single rare variants of large effect size. The first gene mutation associated with AF was found in a 3-generation kindred using linkage analysis and candidate gene screening [1]. A novel missense variant in the *KCNQ1* gene that encodes a voltage-gated potassium (K^+) channel was identified. This discovery

launched a cascade of candidate gene screening studies and numerous variants in other ion channel components of the atrial action potential have been reported (Table 1; reviewed in [2]). Recently, studies in zebrafish have provided new insights into the types of K^+ channels that are active in the heart, demonstrating atrial-specific roles of the two-pore domain K^+ channels, TASK-1 and TWIK-1 [3,4]. Mutation screening of the genes encoding these channels in two cohorts of patients with early-onset and familial AF identified TASK-1 loss-of-function variants in 2 cases [3]. Rare variants in a broad range of non-ion channel genes have now been associated with AF, many of these encoding cardiac transcription factors (Table 1).

Although the list of putative AF "disease genes" continues to grow, it is unclear whether variants in all of these genes are truly causative of AF. Robust genetic evidence for disease association is lacking in all but a handful of these genes, with most variants seen in single cases or in small families that are underpowered for linkage analysis. Moreover, only a few variants in each gene have been described [5,6]. The criteria used to define pathogenicity have evolved over time and many variants previously deemed to be disease-causing mutations would be re-classified using contemporary guidelines [7].

Historically, variant annotation relied on factors such as novelty (as assessed by absence from 100+ healthy control subjects), and disruption of conserved amino acid residues. Interrogation of population sequence databases, such as generated by the Exome Aggregation Consortium (>60,000 subjects), has now revealed that a number of reported cardiomyopathy-associated variants thought to be novel can also be seen in apparently healthy individuals in the general population [8-10]. Functional evaluation of variants has often been limited to bioinformatics predictions that take factors such as sequence conservation into account or *in vitro* analyses, with few examples of animal models that recapitulate human AF. It is now recognised that loss-of-function variants are commonly present in the human

genome [10] and not all of these will be disease-causing. It can be expected that at least some function-altering variants identified in AF patients will be tolerated or have effects that are not directly applicable to AF. The impact of these variants is likely to depend to a large extent on the gene involved and its relative importance to atrial biology. Taken together, these considerations query the extent to which reported variants are *sufficient* alone to cause AF and prompt critical review of the "disease gene" list.

Common variants

In most individuals, AF is complex trait that results from the combined effects of age, and genetic and acquired risk factors. Common variants that modify susceptibility to AF can be identified by genome-wide association studies (GWAS) undertaken in large cohorts of unrelated cases and control subjects. The first major GWAS in AF was reported in 2007, with a significant locus identified in an intergenic non-coding region on chromosome 4q25 [11]. The international AFGen Consortium has been instrumental in advancing this field and subsequent studies undertaken in tens of thousands of subjects have identified a further 13 genetic loci [12,13].

The majority of these 14 AF-associated GWAS loci occur in intergenic regions or in introns and are presumed to contain regulatory sequences that influence gene expression. The GWAS "hits" identify sentinel variants (single nucleotide polymorphisms [SNPs]) that are markers for suites of SNPs that are co-inherited. Further evaluation of each locus is required to identify and characterise potential enhancer or repressor elements and to define function-altering "business" SNPs. The target genes controlled by these regulatory elements may be in close proximity, or located more distally on the same chromosome (*cis*) or on another chromosome (*trans*). Some progress has been made in identifying enhancer elements in the

chromosome 4q25 locus (see Transcription Factors below) but most GWAS loci have yet to be fully explored.

New approaches to variant discovery

Several new strategies have been used to look for both rare and common variants in AF. In contrast to sequencing of single candidate genes, whole-exome sequencing (WES) enables every gene to be assessed. This wealth of additional information magnifies the issue of variant interpretation, especially for genes that have uncharacterized roles in cardiac function. Although WES has been used to identify variants in known disease genes in families with AF [14,15], there have been surprisingly few success stories in "unsolved" cases. Weeke et al. [16] performed WES in 6 AF families, finding 7-15 suspicious rare variants in each of 5 kindreds, none of which clearly co-segregated with AF status, and no variants in one kindred. The authors speculated that variants in non-coding regions could be involved, or that AF in these families might not follow the expected Mendelian model and result from multiple variants. In a population-based WES study, Lubitz et al. [17] compared common and rare coding-sequence variants in 1,734 AF patients and 9,423 subjects without AF. There were no significant associations with AF for >99,000 common variants or for rare variants considered on a *per gene* basis, suggesting that coding-sequence variation accounted for a relatively small proportion of genetically-determined AF.

What have we learned about AF mechanisms?

Ion channel variants

Current concepts for the mechanisms by which genetic variants promote AF are summarised in Figure 1. Studies of ion channel variants have mostly been undertaken in heterologous cell systems with both gain-of-function and loss-of-function effects on channel activity being

identified (reviewed in [2]). Variants that shorten or lengthen atrial action potential duration are thought to predispose to AF by providing a substrate for re-entry or by increasing the propensity for early and/or delayed after-depolarisations, respectively. As more genes are screened in individual cases, it has become apparent that many people carry multiple deleterious rare variants and/or combinations of rare and common variants in ion channel genes [3,6]. *In silico* modelling studies have shown that the functional sequelae of any single variant can be modified by the context, and that different combinations of variants can have epistatic effects that modulate the atrial action potential duration [6]. These findings indicate that unique individual profiles of genetic variants will have different effects on atrial electrophysiological properties. The atrial "environment" can have further modifying influence on channel function, and ion channel variants that have no apparent effects under baseline conditions can profoundly alter channel activity in settings of increased mechanical stress [18].

Transcription factors

A role for transcription factors in the pathogenesis for AF was first brought to attention when *PITX2* was shown to be the closest gene to the chromosome 4q25 GWAS locus [11]. *PITX2* (paired-like homeodomain transcription factor 2) is a transcription factor that belongs to the bicoid class of homeodomain proteins. It is involved in the development of the eye, tooth, and abdominal organs, and mutations in this gene cause Axenfeld-Rieger syndrome, iridogoniodysgenesis syndrome and Peters anomaly. There are 3 isoforms, *PITX2a*, *PITX2b*, and *PITX2c*, with *PITX2c* being the main isoform present in the heart.

The mapping of the chromosome 4q25 locus prompted a series of studies in *Pitx2* knockout mice that established *PITX2* deficiency as a plausible mechanism for AF [19-22]. Finding definitive evidence that *PITX2* is indeed the target gene at this locus has been

challenging. If the GWAS locus does contain a regulatory element for *PITX2*, then it would be expected that expression levels of this gene in heart tissue might be different in SNP carriers and non-carriers. Studies of *PITX2* transcript levels in human heart tissue samples have yielded inconsistent results however, with the largest series (239 subjects) showing no association between SNP status and left atrial *PITX2* expression [21,23]. Further refinement of the chromosome 4q25 locus has revealed that there are four distinct regions that show independent associations with AF [12,24]. Recently, two research groups have identified enhancer elements in the most highly significant of these haplotypes, tagged by the rs2200733 SNP [25,26]. Data showing that these enhancer elements interacted with the *PITX2c* promoter provided the first real evidence that *PITX2* was a potential target gene. Neither of these studies went on to investigate the effects of SNPs on enhancer function. Ye et al. [27] subsequently evaluated the haplotype tagged by the SNP, rs1448818, that is the closest of the 4 GWAS regions to the *PITX2* gene. The authors then identified a regulatory element in this haplotype and showed that it contained a SNP that disrupted a binding site for the transcription factor, TFAP2a. Significant differences in *PITX2c* expression levels were seen in embryonic stem cell-derived cardiomyocytes with SNP-positive cells having relatively lower levels than SNP-negative cells. These findings provide a compelling case for *PITX2* deficiency as a factor contributing to AF in at least one of the four AF susceptibility regions at 4q25. Finally, although mutation screening studies of the *PITX2* gene in cohorts of patients have identified several non-synonymous variants with loss-of-function effects, coding variation in *PITX2* appears to be an uncommon cause of AF [28-30].

How would *PITX2* defects predispose to AF? *PITX2c* has an important role in left-right patterning of the heart, and mice lacking *Pitx2c* show a number of developmental defects including abnormal formation of the sinus node and pulmonary vein myocardial sleeve [31,32]. Several groups have studied the atrial phenotype of heterozygous *Pitx2c*

knockout (+/-) mice, finding altered patterns of gene expression in the left atrium, altered electrophysiological characteristics, and inducible atrial arrhythmias [19-21]. It has been suggested that loss of the normal repression of the sinoatrial node gene program in the left atrium might contribute to this increased arrhythmia propensity. Mice with conditional inactivation of *Pitx2* in the postnatal atrium also exhibit features of sinus node dysfunction as well as impaired atrial conduction. These mice had altered expression of genes encoding ion channels, cell adhesion/cell junction proteins and transcription factors, and showed structural remodelling of intercalated discs [22]. The extent to which these changes might be replicated in human patients who are heterozygous carriers of chromosome 4q25 SNPs remains to be determined. It can be expected that any alterations of *PITX2* expression in SNP carriers might be relatively modest compared to murine models, and that the phenotypic features could be mild. It is also important to bear in mind that regulatory sequences in the chromosome 4q25 locus might also act on target genes in addition to *PITX2*, that are located in *cis* or in *trans*. Elucidating the mechanisms by which chromosome 4q25 SNPs affect AF risk remains an intriguing and ongoing challenge.

Genetic studies have implicated a number of other transcription factors in AF pathogenesis, several of which interact with *PITX2* (Table 1). *GATA-4/5/6*, *NKX2-5*, *NKX2-6*, and *TBX5* are required for differentiation and proliferation of cardiac precursor cells, and mutations in these genes have been associated with a variety of congenital heart defects [33]. There is increasing appreciation that some of these developmental transcription factors might also have roles in mature heart function and in stress-induced hypertrophic remodelling [34]. Selected AF-associated rare variants have been shown to alter transcriptional activity in luciferase assays, but precisely how these rare variants and GWAS SNPs promote atrial arrhythmogenesis remains to be clarified. Taken together, these findings provide a new conceptual framework for consideration of AF risk and suggest that abnormalities of atrial

structure and/or function that arise during key stages of heart development might contribute to a substrate for arrhythmogenesis in adult life.

Myocardial structural components

In recent years, the spectrum of genes implicated in AF has been expanded to include components of myocyte cytoarchitecture, including the sarcomere, cytoskeleton, and nucleus (Table 1). Variants in these genes may give rise to a primary atrial myopathy, as a result of diverse effects on atrial size, contractile function, cell-cell connections and conduction velocity (Figure 1). In addition to mutation-induced primary structural defects, secondary structural remodelling of the atria in patients with chronic AF contributes to arrhythmia maintenance.

Effects of gene expression

The functional sequelae of genetic variants on the encoded protein should not be considered in isolation, and are likely to be dependent on a number of additional intrinsic and extrinsic factors that influence expression of the mutated gene and protein function [35]. The disease process itself can have profound effects on gene expression and widespread changes in the atrial transcriptome have been documented in patients with established AF [36]. Recent data have highlighted a key role for epigenetic mechanisms, including DNA methylation, histone modification and miRNA activity, in orchestrating these gene expression changes [37,38]. Patients with AF may carry mutations in miRNA genes that alter the suites of target mRNAs and transcriptional responses [39].

Impact of genetics on clinical management of AF

Families with AF and early-onset cases

Genetic screening studies of AF families and early-onset AF cases have mainly evaluated small subsets of genes and the potential yield of comprehensive testing all of the AF-associated genes (Table 1) is unknown. However, mutations in the known genes appear to be uncommon and there has not as yet been any major disease gene identified. As a result of this, the expert consensus recommendation for AF is that genetic testing is not indicated as part of routine clinical care [40]. This recommendation can be expected to change in future years as genome-wide approaches to genetic analysis enable more patients to be tested and more genes to be evaluated. Detailed evaluation of cardiac phenotypes of patients with suspected genetic forms of AF may reveal patterns that suggest testing of specific genes. For example, AF may be an isolated finding, or occur in association with sick sinus syndrome, atrial standstill, conduction abnormalities, short/long QT syndrome, and ventricular arrhythmias. Some patients, such as those with *LMNA* mutations, may present with AF and develop dilated cardiomyopathy many decades later. Patients with hypertrophic cardiomyopathy and dilated cardiomyopathy may also be diagnosed with AF, but it can be difficult to determine whether this is part of the genetically-determined phenotype or a consequence of disease. Screening genes involved in ventricular cardiomyopathy may be more useful than screening AF-associated genes in this setting. The potential utility of genetic testing can be considered on a case-by-case basis, but in most situations, the approach to family management involves clinical surveillance to detect asymptomatic arrhythmias, related phenotypic manifestations, and modifiable AF risk factors.

Complex forms of AF

Routine testing of common AF risk SNPs is also not currently recommended [40]. Although highly statistically-significant differences between groups of AF cases and control subjects can be demonstrated for GWAS hits, there is significant overlap between groups and the

value of any single SNP to predict AF susceptibility in an individual patient is limited. A new strategy that has emerged over recent years is assessment of genetic risk scores (GRS) that are based on the total number of variant alleles that a person may carry in sets of GWAS SNPs. In a study >18,000 individuals, Lubitz et al. [41] found that GRS were significantly associated with incident AF, however there was only modest incremental discrimination over clinical AF risk factors. Interestingly, the AF GRS was strongly associated with cardioembolic stroke, confirming the clinical classification of ischemic stroke etiologies and suggesting that AF genetic risk may be a specific marker for strokes cause by cardioembolism. GRS have also been used to predict the severity of AF risk factors such as hypertension and coronary artery disease [42,43], and a GRS for obesity has been used to confirm its causal relationship with AF [44].

Future directions

What is on the horizon for AF genetics? Whole-genome sequencing, not only for affected cases but also on a population level, is no longer a pipedream as governments worldwide formulate strategies for bringing genomic medicine into the public healthcare system. For this enormous mass of sequence information to be clinically useful, intensive research efforts will be needed to characterise the functional effects of genetic variants and to determine their prognostic significance. These studies can be expected to result in new insights into disease mechanisms and the possibility of finding new therapeutic targets. For individual patients, a major challenge will be to generate wholistic models of atrial function that incorporate the total personal burden of rare and common genetic variation, co-morbidities and "environmental" factors. In this regard, phenotypic evaluation of atrial electrophysiological properties may prove to be more informative than identifying single risk parameters. It is possible that some genetic defects may be amenable to specific gene activating/inactivating

therapies and there has been particular interest in the potential use of drugs with atrial-specific actions. Investigation of gene-environment interactions may also reveal subsets of genetically-predisposed individuals in whom aggressive intervention to reduce exacerbating atrial environmental factors is warranted. In 2017 and beyond, understanding the genetic aetiology of AF will have increasing clinical relevance and will play a vital role in achieving personalised approaches to patient care.

Conflicts of interest

Dr. Ellinor is the PI on a grant from Bayer HealthCare to the Broad Institute focused on the genetics and therapeutics of atrial fibrillation. The remaining authors have no disclosures.

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Table 1. Genes associated with AF.

Gene	Protein	AF association	
		Rare variants	GWAS locus*
Ion channels/ion channel-related			
ABCC9	ATP binding cassette transporter; I_{KATP}	X	
HCN4	Pacemaker current; I_F	X	X
JPH2	Intracellular Ca^{2+} signalling	X	
KCNA5	Voltage-gated K^+ channel, Kv1.5; I_{Kur}	X	
KCND3	Voltage-gated K^+ channel, Kv4.3; I_{to}	X	
KCNE1	β-subunit; I_{Ks}, I_{Kr}	X	
KCNE2	β-subunit; I_{Ks}, I_{Kr}	X	
KCNE3	β-subunit; I_{Ks}	X	
KCNE4	β-subunit; I_{Ks}	X	
KCNE5	β-subunit; I_{Ks}	X	
KCNH2	Voltage-gated K^+ channel, Kv11.1; I_{Kr}	X	
KCNJ2	Inwardly rectifying K^+ channel, Kir2.1; I_{K1}	X	
KCNJ5	Inwardly rectifying K^+ channel, Kir3.4; I_{KAch}	X	
KCNJ8	Inwardly rectifying K^+ channel, Kir6.1; I_{KATP}	X	
KCNK3	Two-pore domain K^+ channel, TASK1; I_{leak}	X	
KCNN3	Small conductance, Ca^{2+}-activated K^+ channel, KCa2.2; I_{KCa}	X	X
KCNQ1	Voltage-gated K^+ channel ,Kv7.1;I_{Ks}	X	
RYR2	Ryanodine receptor 2, Ca^{2+} release	X	
SCN1B	β-subunit; I_{Na}	X	
SCN2B	β-subunit; I_{Na}	X	
SCN3B	β-subunit; I_{Na}	X	
SCN4B	β-subunit; I_{Na}	X	
SCN5A	Voltage-gated Na^+ channel, Nav1.5; I_{Na}	X	
Transcription factors			
CUX2	Homeobox protein Cux-2		X
GATA4	GATA-binding protein 4	X	
GATA5	GATA-binding protein 5	X	
GATA6	GATA-binding protein 6	X	
NKX2-5	Homeobox protein NKX2-5	X	
NKX2-6	Homeobox protein NKX2-6	X	
PITX2	Paired-like homeodomain protein 2	X	X
PRRX1	Paired related homeobox protein 1		X
SHOX2	Short stature homeobox protein 2	X	
TBX5	T-box protein 5	X	X
ZFHX3	Zinc finger homeobox protein 3	X	X
Myocardial structural components			
CAV1	Caveolae protein, caveolin 1		X
GJA1	Gap junction protein, connexin 43	X	X
GJA5	Gap junction protein, connexin 40	X	
LMNA	Nuclear envelope protein, lamin A/C	X	
MYH6	Sarcomere protein, myosin heavy chain 6	X	
MYL4	Sarcomere protein, myosin light chain 4	X	
SYNE2	Nuclear envelope protein, spectrin repeat	X	X

	containing nuclear envelope protein 2		
SYNPO2L	Z disc protein, synaptopodin 2-like		X
Signaling, protein turnover, other			
C9orf3	Unknown		X
CAND2	TATA-binding protein, Cullin associated and neddylation disassociated 2		X
GREM2	BMP antagonist, gremlin 2	X	
NEURL	Neutralized E3 ubiquitin protein ligase 1		X
NPPA	Natriuretic peptide precursor A	X	

***** Suspected disease-associated gene in closest proximity to GWAS locus. Statistically significant GWAS loci identified by the international AFGen Consortium are shown. GWAS results from smaller series have not been included.

Figure legends

Figure 1. Schematic showing putative mechanisms by which genetic variants affect the electrical and structural properties of the atria and contribute to a substrate for atrial fibrillation.

Figure
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