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Common Variants in *CASQ2, GPD1L* and *NOS1AP* Are Significantly Associated with Risk of Sudden Death in Patients with Coronary Artery Disease

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Abstract

Background—Recent evidence suggests a genetic component for sudden cardiac death (SCD) in subjects with coronary artery disease (CAD). We conducted a systematic candidate-gene approach using haplotype tagging SNPs (htSNPs) to identify genes associated with SCD risk in the context of CAD.

Methods and Results—We investigated 1,424 htSNPs representing 18 genes with mutations described in patients with ventricular arrhythmias, in 291 subjects from the Oregon Sudden Unexpected Death Study (Ore-SUDS). The Ore-SUDS is an ongoing prospective investigation of SCD in the Portland, OR metropolitan area (pop. 1,000,000). SCD cases were ascertained from multiple sources and medical records were reviewed to determine the presence of CAD. A total of 36 SNPs were associated with risk of SCD (uncorrected p-values <0.01) in the initial study sample. These SNPs were subsequently tested for replication in an independent case-control study sample from the Ore-SUDS (n=688). The association analysis in the replication stage revealed six SNPs associated with SCD: CASQ2 region (rs17500488; P=0.04, rs3010396; P= 0.007, rs7366407; P=0.04), NOS1AP (rs12084280; P=0.04, rs10918859; P=0.02) and one SNP located ~26 kb upstream of GPD1L (rs9862154; P= 0.04).

Conclusions—Common variations in or near *CASQ2, GPD1L* and *NOS1AP* are associated with increased risk of SCD in patients with CAD. These findings provide further evidence for overlap

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between the genetic architecture of rare and common forms of SCD, and replication in additional populations is warranted.

Keywords

death; sudden; risk; prediction; genomics; variants

Introduction

Sudden cardiac arrest leading to sudden cardiac death (SCD) is a major cause of mortality in the US, accounting for 250,000–300,000 deaths on an annual basis [1]. Prediction and prevention have been recognized as pivotal steps toward improved outcomes [1], particularly since national rates of survival from sudden cardiac arrest are below 5%. Since the vast majority of SCD cases (at least 80%) have evidence of associated severe coronary artery disease [2], the latter phenotype has become the focus of intensive investigation.

Several studies have highlighted the evidence for a clear genetic contribution in the more common SCD phenotype among patients with coronary artery disease [3–6]. We have recently published results from ongoing genome-wide association studies of SCD, identifying novel loci associated with either protection from [7], or susceptibility to SCD [8]. However, much needs to be learned regarding the genetic architecture of common, complex forms of SCD[9]. On the other hand, due to multiple kindred-based investigations performed in the last two decades, significant knowledge has accumulated regarding gene defects that cause rare primary arrhythmia syndromes. Several mutations in multiple genes have been identified in inherited forms of the long and short QT syndromes [10–14]. Brugada syndrome is characterized by idiopathic ventricular fibrillation and characteristic ECG changes [15–17]; and catecholaminergic polymorphic ventricular tachycardia (CPVT) is a familial arrhythmogenic disorder manifesting with ventricular tachyarrythmias [18, 19]. However, primary arrhythmia syndromes account for only a small proportion of SCD cases in the general population. We hypothesized that variations in genes that cause primary arrhythmia syndromes could be associated with the more common, complex SCD phenotype observed in patients with CAD. Given that candidate gene-based evaluations can be complementary to genome-wide association efforts, we conducted a systematic candidate-gene SNP genotyping, case-control study of SCD in CAD subjects, based on common variations among genes known to cause primary arrhythmia disorders.

Methods

Clinical diagnosis

The Oregon Sudden Unexpected Death Study (Ore-SUDS) is an ongoing population based study of SCD in residents of Portland, OR and the surrounding metropolitan area [7,20–25]. SCD was defined as a sudden unexpected pulseless condition of likely cardiac etiology; survivors of sudden cardiac arrest were included. If the event was unwitnessed, subjects were required to have been seen living and symptom free within 24 hours of sudden death. A diagnosis of SCD was assigned following in-house adjudication by three physicians who evaluated arrest circumstances and all available clinical data. Exclusion criteria for Ore-SUDS SCD cases were chronic terminal illness, and non-cardiac causes of sudden death such as pulmonary embolism, cerebrovascular event, traumatic death or drug overdose.

Subject selection

Case subjects in this analysis were individuals with SCD who were white non-Hispanic adults (age ≥ 18 years) with DNA for analysis. Control subjects were white, non-Hispanic

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individuals with medically documented coronary artery disease, and without prior history of sudden cardiac arrest or ventricular arrhythmias. They were recruited from individuals transported by the Emergency Medical Response system for complaints suggestive of ongoing coronary ischemia, from clinics of participating health systems, or from patients who had received a coronary angiogram revealing significant CAD. After consent was obtained, medical records for each potential control subject were reviewed; those with documented CAD (as defined below) were enrolled.

Documented CAD was defined as ≥ 50% stenosis of a major coronary artery on angiogram or postmortem examination; history of percutaneous coronary intervention (PCI) or coronary artery bypass grafting (CABG); physician report of MI; pathologic Q waves on ECG; or myocardial infarction (MI) history determined by any two of the following three: ischemic symptoms, ECG changes, or positive troponins/CKMB. All controls had documented CAD. A total of 346 SCD cases (52%) had documented CAD by autopsy or medical records. Ninety-four percent of cases had either medically-documented coronary artery disease (CAD), as defined below, or had presumed CAD based on previous studies that have reported that the vast majority (85–95%) of subjects with SCD at age ≥ 50 years have significant coronary disease at autopsy [2,26].

Blood samples were obtained for SCD cases from the first responders during attempted resuscitation or from the medical examiner, when autopsies were performed. Samples were obtained from control subjects at the time of their visit to the study site for a blood draw and ECG. All aspects of this study were approved by the appropriate institutional review boards.

Candidate-Based Genotyping and SNP selection

We performed a custom SNP genotyping assay on the initial study population using a candidate-gene-based approach with the GoldenGate™ assay (Illumina, Inc., San Diego, CA). SNPs representing 18 high priority genes were selected for analysis. Genes were considered to have a high priority if mutations had been described in patients with primary ventricular arrhythmia syndromes or if these were genes encoding crucial subunits of such candidate genes. A list of the selected genes is shown in Table 1.

The common genetic variation of each gene was covered by systematic selection of haplotype tagging SNPs (htSNPs), considering both intronic and exonic variants. SNP selection was performed in mid 2007 and was performed using the software tagger [27] based on the HapMap data release #20 / phase II from January 2006 using the NCBI B35 genome assembly and dbSNP b125 data applying the following criteria: HapMap CEU population, pairwise tagging only with a cut-off of $r^2 \geq 0.8$ and a minor allele frequency (MAF) of at least 10%. To account for genetic variation in genome regions surrounding each gene, up- and downstream genetic information was included in the tagging procedure. The respective regions were defined using linkage-disequilibrium (LD) blocks as described elsewhere [28]. If the detected LD-blocks were smaller than 50kb, then at least 50 kb of both up- and downstream information was tagged. Due to the potentially higher a priori probability of being a pathophysiologically causal variant, all known nonsynonymous coding variants in the selected genes were added to the assay design.

SNP genotyping

A SNP genotyping assay containing all mentioned variants was purchased from Illumina based on the Illumina GoldenGate™ technology. Genotyping was performed according to the manufacturer's recommendations using the Illumina Beadstation 500G. Illumina's BeadStudio 3.1.14 genotyping module was used to automatically cluster, call genotypes, and

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assign confidence scores. All markers with call frequency lower than 95% were manually edited.

SNP Validation

SNPs significantly associated with risk of SCD were subsequently validated on a different set of cases and controls. These samples from the Ore-SUDS study were also white Non-Hispanic from the same geographic area as the original sample and ascertained according to the subject selection characteristics listed above. Genotyping for the replication stage was performed using PCR, iPLEX single base primer extension and subsequent MALDI-TOF mass-spectrometry on a Sequenom platform (Sequenom, San Diego, CA) according to the manufacturer's standard recommendations. Genotypes were determined using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) on a MASSArray Compact system and analyzed using the software TyperAnalyzer (Sequenom). A total of 40 SNPs were processed in two experiments of 30 and 10 variants, respectively. Replicated associations with a Pearson's Chi-square p-value <0.05 were considered truly associated with the phenotype.

SNP *rs9862154* did not meet the call rate cutoff in the iPLEX and was genotyped in a single ABI TaqMan Genotyping assay according to the manufacturer's directions (Applied Biosystems, Inc.).

Statistical analysis

Association analyses were performed on the original study population and the validation dataset using PLINK (http://pngu.org/~purcell/plink/ [29]). SNPs were tested for genotype-phenotype association using the genotypic C/C association test in PLINK, which calculates the association of genotype to SCD using a full model of inheritance. Asymptotic p-values are provided for each of four association tests: additive, genotype, dominant and recessive.

Results

Genotyping in the discovery population

A panel of 1,424 SNPs was genotyped in 291 individuals. The average call rate was 96%. Our initial study population consisted of 141 cases (43 female and 98 male) and 150 controls (48 female and 102 male). Ten individuals missing >10% genotype data were removed from analysis. The sample remaining for analysis consisted of 134 cases (mean age 66±14 yrs, 71% male) and 147 controls (mean age 66±13 yrs, 67% male) (Table 2).

Of 1,424 SNPs, 50 SNPs were missing >10% of genotypes and 67 SNPs had a minor allele frequency (MAF) of < 0.01, which resulted in exclusion of a total of 76 SNPs from the dataset. SNPs not in Hardy-Weinberg equilibrium ($P < 0.001$) in control subjects (n=14) were removed from the study because their inclusion could lead to false signals of association [30].Thus, 281 subjects with genotypes in 1,334 SNPs (overall call rate of 99.64%) were included in the final dataset. Thirty-eight SNPs were associated with SCD (uncorrected p-value <0.01) in at least one of the association models (Table 3), while 53 significant associations would have been expected by chance alone.

Genotyping in the validation population

In the second stage, we performed validation genotyping for these 38 SNPs in an independent Ore-SUDS sample (n=688). Replicated associations with a Pearson's Chi-square p-value statistic of less than 0.05 were considered truly associated with the phenotype. Several SNPs showed association with SCD on chromosome 1, near the *CASQ2* gene (just upstream of *NOS1AP*) and in the *NOS1AP* gene (nitric oxide synthase 1 adaptor

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protein) under different genetic models. In addition, the SNP *rs9862154* ~26 kb upstream of *GPD1L* was associated with SCD under the recessive genetic model (Table 3).

Discussion

In the present study, we observed and validated significant associations between DNA variants located in non-coding regions of *CASQ2, GPD1L* and *NOS1AP* genes, and risk of SCD in subjects with CAD. However, false positive results should be evaluated in future, larger replication efforts. *CASQ2* and *GPD1L* are of special interest due to their known involvement in the primary arrhythmia syndromes and *NOS1AP* has been previously associated with prolongation of the QT interval and risk of SCD in the community. These findings indicate the interesting possibility of overlap between the genetic architecture of rare and common forms of SCD.

CASQ2 encodes the intra-sarcoplasmic reticulum Ca^{2+} binding protein cardiac calsequestrin. Mutations in *CASQ2* have been associated with CPVT, a rare familial arrhythmogenic disorder characterized by malignant ventricular arrhythmias [31–33]. *GPD1L* can harbor coding mutations among kindreds with the Brugada syndrome. An interesting relationship has also been described between *GPD1L* and the *SCN5A* gene, implicated in the majority of causative mutations discovered for Brugada syndrome. London, Dudley and colleagues have shown that missense mutations in *GPD1L* cause reduced trafficking of the cardiac Na+ channel to the cell surface, reducing inward Na+ current, and causing Brugada syndrome [34]. Furthermore, the downregulation of Na current by *GPDIL* mutations is likely due to alteration of the oxidized to reduced Nicotinamide adenine dinucleotide hydrogenase [NAD(H)] balance [35]. In recent work, Valdivia *et al.* [36] lend support for linking mutations in GPD1L to SCD using an *in vitro* cell culture system expressing GPD1L and SCN5A mutant and wildtype constructs. They demonstrated association of GPD1L with SCN5A; mutants of GPD1L increased PKC-mediated phosphorylation of SCN5A which in turn causes a dysfunction in sodium current, a mechanism for ventricular arrhythmias.

NOS1AP encodes a nitric oxide synthase 1 adaptor protein. Common variants in *NOS1AP* have been associated with prolongation of the QT interval [37–44] as well as increased risk of SCD [45–46]. Kao et al reported that two non-correlated *NOS1AP* SNPs (*rs16847548* and *rs12567209*) were associated with SCD in a large US community [46].The SNP *rs12567209* is in high linkage disequilibrium (LD) with *rs12084280* reported in the present study (D' = 1.0; r^2 =0.92). Of note, a NOS1AP variant was also identified as a risk modifier among patients with familial long QT syndrome [47–48]. Although it is well documented that *NOS1AP* common variants are associated with increased risk of SCD, the specific functional role of *NOS1AP* variants merits further evaluation.

Whereas mutations have been described and characterized in *CASQ2* earlier, in the present study we report a role for common variants for which functional evaluation has yet not been performed. One hypothesis might be that common SNPs are markers of functional, rare variants that are not covered by current genotyping strategies, similar to what has been shown for hypertriglyceridemia [49]. For the elucidation of the relationship between common and rare variants at a single locus and to identify pathophysiologically causal variants, the current advent of high-throughput sequencing efforts is a promising strategy for the near future.

Limitations

Our sample size is relatively small, and the possibility exists that we have missed additional association signals. Future investigation in larger cohorts will be needed to detect such associations with sufficient statistical power. However, this is a challenging phenotype to

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study in the community and subjects were matched for presence of CAD. Furthermore, our cases and controls were all derived from the same underlying population and adjudicated following a common, standardized protocol. All tested genes bear a high a priori probability for a true associations based on previous reports on their pathophysiological involvement in our phenotype. A Bonferroni correction for multiple testing is often applied in genome-wide association studies, but might be considered too conservative for highly selective candidate gene-based approaches. Independent replication of significant findings can be regarded as the most reliable form of validation. We therefore did not perform correction for multiple testing in association results in either the discovery or the replication population, but rather attempted independent replication.

Conclusions

These findings suggest that common variants in genes previously implicated in relatively rare inherited forms of arrhythmias may contribute to the pathogenesis of more common, complex forms of SCD. Further studies in larger samples are warranted to validate the contribution of these genes in SCD.

Sudden cardiac death remains a public health problem of significant magnitude and the key to prevention is improvement in risk stratification methodology. Recent studies have shown that there is evidence of a genetic component even among patients with coronary disease who suffer sudden cardiac death, the most common yet complex form of this condition. We employed high through-put genetic analysis to evaluate the potential role of genes that are known to be causative in more rare, familial forms of sudden cardiac death, such as the long QT and Brugada syndromes. The results indicate that common variations in the genes known to be involved in the rare syndromes are also associated with sudden cardiac death in the more common and complex coronary artery disease manifestation. These findings provide evidence for a unifying genetic link between rare and common forms of sudden cardiac death, and is likely to inform the development of enhanced risk stratification methodologies.

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Table 1

Candidate genes under investigation and the number of tag SNPs genotyped.

Gene	Chr	Position	Region included	# SNPs
AKAP9 Isoform 1	7	91570192–91739989	9330760–9396760	20
ANK 2 Isoform 1	4	113970870–114304885	113802396–114332396	118
CACNA1C	12	2162464–2802107	2029739–2949739	176
NOS1AP (CAPON)	1	162039581–162339813	161948342–162438342	130
CASQ2	1	16209379–116311270	116097958–116417958	70
CAV3	3	8775496–8788450	8725000–8865000	63
FKBP1B	2	24272628–24286548	24222627–24336547	13
GPD1L	3	32148144–32210201	32004996–32254996	50
KCNE1	21	35818988–35883613	35718130–36008130	71
KCNE2	21	35736323–35743440	35718130–36008130	28
KCNH2 / HERG	7	150642049–150675014	50152739–150762352	67
KCNJ2	17	68165676–68176181	68098405–68253405	42
KCNQ1	11	2466221–2870339	2303424–2943424	151
RYR2	1	237205702–237997288	237101177–238101176	240
SCN1B	19	35521534–35531352	35448160–35578160	24
SCN4B	11	118004092–118023535	117844790–118114790	54
SCN4A	17	62015914–62050278	61866268–62166268	25
SCN5A	3	38589553–38691163	33867996–38814996	82
Total				1,424

All data based on hg 19, NCBI build 37.

Chr, chromosome.

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Table 2

Demographics in original and validation samples.

	Cases	Controls
Original Sample		
N (281 total)	134	147
% Male	71%	67%
Age (Mean, SD)	66 (14)	66 (13)
Validation Sample		
N (688 total)	536	152
% Male	71%	66%
Age (Mean, SD)	62 (15)	666 (11)

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Table 3

SNPs significantly associated (p< 0.01) with SCD.

CHR	SNP	MAF	GENE	MODEL	p-value*	Validation p-value†
1	rs17500488	0.095	near CASQ2 (VANGL1)	ADDITIVE	0.0041	**0.04**
1	rs7536370	0.363	CASQ2	DOM	0.0096	0.800
1	rs3010396	0.454	CASQ2	GENO	0.0208	**0.007**
1	rs11586273	0.329	near CASQ2 (LOC400769)	ADDITIVE	0.0053	0.120
1	rs7366407	0.286	near CASQ2 (LOC400769)	DOM	0.0023	**0.040**
1	rs12084280	0.11	NOS1AP	GENO	0.0028	**0.040**
1	rs12567209	0.103	NOS1AP	GENO	0.0091	0.180
1	rs4656355	0.4	NOS1AP	DOM	0.0033	0.710
1	rs12026452	0.156	NOS1AP	DOM	0.0089	0.510
1	rs7412698	0.345	NOS1AP	DOM	0.0048	0.820
1	rs10918859	0.194	NOS1AP	DOM	0.0047	**0.020**
1	rs4531275	0.322	NOS1AP	DOM	0.0001	0.220
1	rs3924139	0.345	NOS1AP	DOM	0.0003	0.330
1	rs4424487	0.361	NOS1AP	DOM	0.0003	0.370
1	rs10918936	0.388	NOS1AP	DOM	0.0023	0.860
1	rs4657178	0.244	NOS1AP	DOM	0.0030	0.720
1	rs10918963	0.203	NOS1AP	DOM	0.0048	0.180
1	rs10753784	0.421	NOS1AP	DOM	0.0045	0.400
1	rs12733377	0.454	NOS1AP	DOM	0.0100	0.570
1	rs12048222	0.255	NOS1AP	GENO	0.0036	0.720
1	rs1881548	0.201	RYR2	ADDITIVE	0.0084	0.470
1	rs6678625	0.149	RYR2	ADDITIVE	0.0014	0.340
1	rs888438	0.173	RYR2	ADDITIVE	0.0041	0.220
1	rs10158497	0.27	RYR2	DOM	0.0060	0.790
1	RYR2_A1136V	0.012	RYR2, exon 28	ADDITIVE	0.0050	0.430
3	rs4955135	0.205	GPD1L	REC	0.0063	0.150

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CHR	SNP	MAF	GENE	MODEL	p-value*	Validation p-value†
3	rs9862154	0.222	GPD1L	REC	0.0019	**0.044**
4	rs4627864	0.386	ANK2	REC	0.0077	0.560
4	rs2107026	0.158	ANK2	DOM	0.0047	0.070
4	rs29308	0.217	ANK2	REC	0.0062	0.880
7	rs3918227	0.101	NOS3	DOM	0.0072	0.320
11	rs7104786	0.373	ASCL2; C11orf21	DOM	0.0027	0.240
11	rs10766212	0.443	KCNQ1	DOM	0.0031	0.500
11	rs8234	0.329	KCNQ1, 3'UTR	REC	0.0030	0.080
11	rs10502228	0.196	SCN4B; MPZL3	REC	0.0056	0.950
12	rs1558322	0.278	CACNA1C	GENO	0.0046	0.760
21	rs2247810	0.36	KCNE1	DOM	0.0018	0.700
21	rs1547356	0.273	KCNE1	REC	0.0044	0.990

* p-value from additive, dominant, recessive, or genotypic model.

† Validation p-values are shown in bold if less than or equal to 0.05.

CHR – chromosome; ADDITIVE – additive genetic model; DOM – dominant genetic model; GENO – genotypic model; REC – recessive genetic model. MAF – minor allele frequency in all subjects.