



Genetic variability of *RyR2* and *CASQ2* genes in an Asian population

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ARTICLE INFO

Article history:

Received 19 June 2008

Received in revised form 20 March 2009

Accepted 28 July 2009

Available online 25 August 2009

Keywords:

Cardiac arrhythmia

Ryanodine receptor

Calsequestrin

Polymorphisms

Sudden unexplained death

ABSTRACT

We analyzed the coding regions of the cardiac calcium-handling genes, ryanodine receptor 2 (*RyR2*) and calsequestrin 2 (*CASQ2*) for genetic variants in a healthy Chinese population ($n = 95$) and in a cohort of 28 sudden unexplained death victims. Mutations in *RyR2* and *CASQ2* have been shown to alter calcium homeostasis during excitation–contraction coupling and predispose individuals to fatal cardiac arrhythmias. The genetic screening was accomplished by denaturing high-performance liquid chromatography and DNA sequencing methods. Genetic analysis revealed the following non-synonymous genetic variations: two reported *RyR2* polymorphisms; 5654G>A (G1885E) and 5656G>A (G1886S), two reported *CASQ2* polymorphisms; 196A>G (T66A) and 226G>A (V76M) and one novel *CASQ2* mutation; 529G>C (E177Q). The functional significance of the novel *CASQ2* mutation has not been evaluated and characterized. This study shows that multiple genetic variations of the *RyR2* and *CASQ2* genes exist in the two study populations. The inter-individual genetic variability may underlie the different susceptibility of individuals to developing ventricular tachycardia. The research results will be valuable for which future work involving clinical and forensic samples can be based upon to distinguish potential disease-associated mutations from common polymorphisms.

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1. Introduction

The sudden death syndrome is the event of a sudden and unexpected death occurring within minutes after collapse. Most of these sudden deaths are associated with existing ischaemic heart diseases which are easily detectable during post-mortem examination. Of particular concern have been the occurrences of sudden deaths in apparently normal healthy adults during conditions of stress or strenuous physical activities. This group constitutes only a minority of all sudden deaths. The term sudden unexplained death syndrome (SUDS) has been applied to such sudden unexplainable natural deaths involving healthy individuals with no clear cause indicated.

Cardiac arrhythmias play an important role in the pathogenesis of SUDS. These cardiac syndromes include, amongst others, long QT syndrome, Brugada syndrome and catecholaminergic polymorphic ventricular tachycardia (CPVT). Mutations in calcium-handling genes have been associated with CPVT. A substantial amount of evidence has suggested that mutant calcium-handling genes, cardiac ryanodine receptor (*RyR2*) and calsequestrin (*CASQ2*), are involved in abnormal regulation of intracellular Ca^{2+} , which plays a key role in the development of fatal ventricular

arrhythmias during increased levels of catecholamines or β -adrenergic stimulation [1,2].

The *RyR2* gene comprises 105 exons encoding an mRNA of approximately 15 kb [3]. It is a cardiac sarcoplasmic reticulum (SR) calcium release channel which involves in the release of Ca^{2+} from the SR stores during excitation–contraction (EC) coupling via a mechanism known as Ca^{2+} induced Ca^{2+} release [4,5]. The *CASQ2* gene comprises 11 exons encoding a precursor protein of 399 amino acids. It is a low affinity, high capacity Ca^{2+} binding protein whose primary function is to store Ca^{2+} within the SR [6].

This study focuses on the genetic aspects of sudden unexplained death associated with no known causes of death at autopsy. In particular, sudden unexplained deaths which occur during stress-related or increased physical activities may be attributed to CPVT. We performed genetic screening of *RyR2* and *CASQ2* genes in a cohort of 28 sporadic sudden unexplained death subjects and the Singaporean Chinese population. This is the first report on the comprehensive analysis of *RyR2* and *CASQ2* genes in the Asian population.

2. Materials and methods

2.1. Study population

Whole blood (10 ml) or frozen heart tissue samples from 28 sudden death subjects <35 years old were obtained from the Forensic Medicine Division, Health Sciences Authority (HSA), Singapore. The post-mortem examination revealed no cardiovascular abnormality or significant autopsy findings that might indicate a

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clear cause of death. Thirteen of the sudden death subjects collapsed while engaging in vigorous physical activity. The contribution of emotional stress to the remaining 15 non-exercise related deaths cannot be ruled out. The study was approved by the Institutional Review Board, HSA, Singapore.

Genetic materials were randomly selected from a previously developed cell repository from healthy volunteers of Chinese descent. The fully anonymized white cell lines developed from 95 Chinese individuals of the Singaporean population, aged 18–48 years old (median age = 22) with 1:1 male to female ratio, were screened. All donors had been recruited previously in accordance with requirements of the ethical review board of the National University Hospital, Singapore and provided written informed consent. Ethnicity was defined through self-declaration by the donors of similar ethnicity through three generations.

2.2. Genetic analysis

For the sudden unexplained death subjects, genomic DNA was extracted either from blood or tissue samples using standard desalting methods. The exonic fragments for the *RyR2* and *CASQ2* genes were generated using primer pairs obtained from Tiso et al [1] and http://mutation.swmed.edu/ex-lax/ELXRdb/NM_001232, respectively. The fragments were amplified by PCR and then subjected to sequencing using BigDye Terminator v3.1 Cycle Sequencing kit and run on ABI 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

For the Chinese population, a targeted genetic analysis of 54 exons (Exons 2–20, 37, 39–49, and 83–105) in *RyR2* was performed by denaturing high performance liquid chromatography (Transgenomic WAVE Nucleic Acid Fragment Analysis System; Omaha, NE, USA) and DNA sequencing as described previously [7]. The exonic fragments of *CASQ2* were directly sequenced.

The genetic variations were classified according to genotype and their corresponding frequencies calculated. These observed frequencies were then compared with the expected frequencies as predicted by the Hardy–Weinberg equilibrium using chi-square test analysis. Comparison of the allelic frequencies between the populations was also analyzed by chi-square test. Statistical significance was considered at $p < 0.05$.

3. Results and discussion

We identified three *CASQ2* polymorphisms and one *CASQ2* mutation; two reported non-synonymous single nucleotide polymorphisms (SNPs), one reported synonymous SNP, and one novel mutation (Table 1). The 196A>G (T66A) polymorphism was commonly detected in both the Chinese and sudden unexplained death populations and probably represents an innocent polymorphism. In contrast, the 226G>A (V76M) variant was a less common polymorphism detected only in the Chinese population. The 196A>G allele frequency is significantly lower in the Finnish population [8] compared to our Chinese population ($p < 0.05$). No significant difference in allele frequency was observed between these two populations for the 226G>A polymorphism. The V76M variant has demonstrated functional defects such as diminished calcium binding, altered monomer polymerization and dimer formation, however no functional changes have been reported for the T66A variant [9].

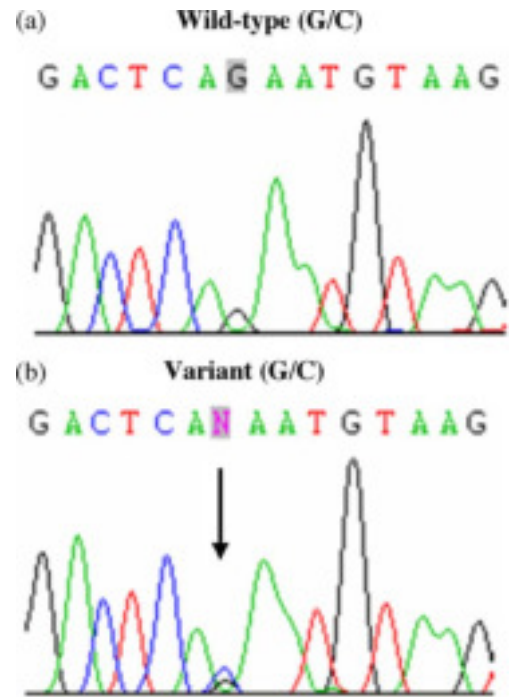


Fig. 1. Direct sequencing of PCR-amplified genomic DNA from the Chinese population revealed a novel heterozygous mutation 529G>C. It demonstrated GAA>CAA mutation, affecting the glutamic acid residue at position 177, which was replaced by a glutamine in domain II of *CASQ2* protein. Electropherograms showing (a) wild-type and (b) variant sequences. Arrow indicates the variant nucleotide position.

The novel variant, 529G>C (E177Q) of *CASQ2* was found in a single normal Chinese subject (Fig. 1). It was not present in our Malay, Indian, Japanese and Caucasian populations ($n = 95$ each). The novel variant results in the exchange of a negatively charged glutamic acid to a neutral glutamine at amino acid position 177 (E177Q). The E177 residue lies in domain II of *CASQ2* and multiple sequence alignment shows the residue is fully conserved across all species of *CASQ2* and *CASQ1*, suggesting the importance of this amino acid in protein function (Fig. 2). The substitution may disrupt the normal chelation function and alter Ca^{2+} binding capacity of domain II. Using the PolyPhen program (<http://genetics.bwh.harvard.edu/pph/>) to predict the functional effects of the amino acid substitution, 529G>C (E177Q) was however predicted to be benign based on the position-specific independent

Table 1

Genotype distributions and allele frequencies of *CASQ2* polymorphisms in 123 DNA samples of Asian individuals.

Location	Variant ^a	Amino acid change	SNP ID dbSNP (NCBI)	Distribution of genotypes		Allele frequency	
				Sudden death ($n = 28$)	Chinese ($n = 95$)	Sudden death ($n = 28$)	Chinese ($n = 95$)
Exon 1	196A>G	T66A	rs4074536	A/A: 5 A/G: 19 G/G: 4	A/A: 21 A/G: 54 G/G: 20	0.482	0.495
Exon 1	226G>A	V76M	rs10801999	G/G: 28 G/A: 0 A/A: 0	G/G: 88 G/A: 7 A/A: 0	0	0.037
Exon 4	529G>C	E177Q ^b	–	G/G: 28 G/C: 0 C/C: 0	G/G: 94 G/C: 1 C/C: 0	0	0.005
Exon 11	1185C>T	D395D	rs7413162	C/C: 10 C/T: 15 T/T: 3	C/C: 45 C/T: 44 T/T: 6	0.375	0.295

^a Nucleotide number relative to the translation start site.

^b Novel mutation detected in this study.

Table 2
Genotype distributions and allele frequencies of RyR2 polymorphisms in 123 DNA samples of Asian individuals.

Location	Variant ^a	Amino acid change	SNP ID dbSNP (NCBI)	Distribution of genotypes		Allele frequency	
				Sudden death (n=28)	Chinese (n=95)	Sudden death (n=28)	Chinese (n=95)
Exon 37	5654G>A	G1885E	rs41315858	G/G: 27 G/A: 1 A/A: 0	G/G: 95 G/A: 0 A/A: 0	0.018	0
Exon 37	5656G>A	G1886S	rs3766871	G/G: 25 G/A: 3 A/A: 0	G/G: 82 G/A: 13 A/A: 0	0.054	0.068

^a Nucleotide number relative to the translation start site.

CASQ2_HUMAN_MUTANT/1-399	YIKLIGFFKSEDS	YYKAFEEAAEHFQP
O14958 CASQ2_HUMAN/1-399	YIKLIGFFKSEDS	YYKAFEEAAEHFQP
P51868 CASQ2_RAT/1-413	QIKLLGFFKNEDS	YYKAFQEAAEHFQP
O09161 CASQ2_MOUSE/1-415	QTKLLGFFKNEDS	YYKAFQEAAEHFQP
Q5RAN9 CASQ2_PONP/1-400	YIKLIGFFKSGDS	YYKAFEEAAEHFQP
P31235 CASQ2_RABBIT/1-409	HIKLIGFFKSADS	YYKAFEEAAEHFQP
P12637 CASQ2_CANIS/1-410	QIKLLGFFKSEES	YYKAFEEAAEHFQP
P91413 CASQ1_HUMAN/1-390	EIKLIGYFKSKDS	HYKAFEDAAEEFHP
P19204 CASQ1_CHICK/1-406	EIKLIGYFKGEDS	HYKAFEEAAEHFQP
P07221 CASQ1_RABBIT/1-395	EIKLIGYFKKDS	HYKAFKEAAEEFHP
P31231 CASQ1_RAN/1-420	EPKLLIGYFKNEDS	HYKAYEDAAEEFHP

Fig. 2. Multiple sequence alignment of a segment of the human CASQ2 protein sequence with the corresponding segments in other homologues. The protein sequences were obtained from Uniprot (<http://www.ebi.uniprot.org/index.shtml>) protein database. The first sequence in the entry indicates the mutated human CASQ2 and the highlighted column indicates the E177Q mutated position. The Uniprot accession numbers of the aligned proteins are as follows: human cardiac CASQ2, O14958; rat cardiac CASQ2, P51868; mouse cardiac CASQ2, O09161; orangutan cardiac CASQ2, Q5RAN9; rabbit cardiac CASQ2, P31235; dog cardiac CASQ2, P12637; human cardiac calsequestrin 1 (CASQ1), P31415; chicken cardiac CASQ1, P19204; rabbit cardiac CASQ1, P07221; frog cardiac CASQ1, P31231.

count score differences derived from multiple alignments. The prediction of the mutation on the final protein based on location, computational modeling, and physicochemical properties of the amino acids is only speculative and the exact functional consequence demands further experimental studies.

Genetic analysis of the RyR2 exons revealed two reported non-synonymous substitutions, and 15 synonymous SNPs (Table 2). 5454G>A (Gly1885Glu) was detected only in a sudden unexplained death sample while 5656G>A (Gly1886Ser) was present in both populations. The composite polymorphisms were reported in German patients with arrhythmogenic right ventricular cardiomyopathy and the combined expression was associated with increased diastolic channel activity leading to SR Ca²⁺ leak [10].

This study shows the existence of multiple genetic variations of the RyR2 and CASQ2 genes in the Asian population. The research findings from this study will be valuable for which future work involving forensic and clinical samples can be based upon to distinguish potential disease-associated mutations from common

polymorphisms in forensic investigations and disease association studies.

Acknowledgments

This study was supported by grants from the Agency for Science, Technology and Research, Biomedical Research Council, Singapore, Singapore Heart Foundation, Singapore, and Academic Research Fund, National University of Singapore, Singapore.

References

- [1] X.H. Wehrens, S.E. Lehnart, F. Huang, J.A. Vest, S.R. Reiken, P.J. Mohler, J. Sun, S. Guatimosim, L.S. Song, N. Roseblit, J.M. D'Armiento, C. Napolitano, M. Memmi, S.G. Priori, W.J. Lederer, A.R. Marks, FKBP12,6 deficiency and defective calcium release channel (ryanodine receptor) function linked to exercise-induced sudden cardiac death, *Cell* 113 (2003) 829–840.
- [2] S.E. Lehnart, X.H. Wehrens, P.J. Laitinen, S.R. Reiken, S.X. Deng, Z. Cheng, D.W. Landry, K. Kontula, H. Swan, A.R. Marks, Sudden death in familial polymorphic ventricular tachycardia associated with calcium release channel (ryanodine receptor) leak, *Circulation* 109 (2004) 3208–3214.
- [3] R.E. Tunwell, C. Wickenden, B.M. Bertrand, V.I. Shevchenko, M.B. Walsh, P.D. Allen, F.A. Lai, The human cardiac muscle ryanodine receptor calcium release channel: identification, primary structure and topological analysis, *Biochem. J.* 318 (1996) 477–487.
- [4] A. Fabiato, Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell, *J. Gen. Physiol.* 85 (1985) 247–289.
- [5] D.M. Bers, Cardiac excitation–contraction coupling, *Nature* 415 (2002) 198–205.
- [6] K. Yano, A. Zarain-Herzberg, Sarcoplasmic reticulum calsequestrins: structural and functional properties, *Mol. Cell. Biochem.* 135 (1994) 61–70.
- [7] A. Bagattin, C. Veronese, B. Bauce, W. Wuyts, L. Settimo, A. Nava, A. Rampazzo, G.A. Danielli, Denaturing HPLC-based approach for detecting RyR2 mutations involved in malignant arrhythmias, *Clin. Chem.* 50 (2004) 1148–1155.
- [8] P.J. Laitinen, H. Swan, K. Kontula, Molecular genetics of exercise-induced polymorphic ventricular tachycardia: identification of three novel cardiac ryanodine receptor mutations and two common calsequestrin 2 amino-acid polymorphisms, *Eur. J. Hum. Genet.* 11 (2003) 888–891.
- [9] E. Kim, B. Youn, L. Kemper, C. Campbell, H. Milting, M. Varsanyi, C. Kang, Characterization of human cardiac calsequestrin and its deleterious mutants, *J. Mol. Biol.* 373 (2007) 1047–1057.
- [10] H. Milting, N. Lukas, B. Klauke, R. Korfer, A. Perrot, K.J. Osterziel, J. Vogt, S. Peters, R. Thieleczek, M. Varsanyi, Composite polymorphisms in the ryanodine receptor 2 gene associated with arrhythmogenic right ventricular cardiomyopathy, *Cardiovasc. Res.* 71 (2006) 496–505.