Author's response to reviews

Title: Search for Cardiac Calcium Cycling Gene Mutations in Familial Ventricular Arrhythmias Resembling Catecholaminergic Polymorphic Ventricular Tachycardia

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Version: 2 Date: 10 November 2008

Author's response to reviews: see over
RE: Manuscript number 1051311299208940, a revised version

Dear Dr Norton,

Thank you for your letter of August 22nd, 2008, indicating that our manuscript entitled “Search for Cardiac Calcium Cycling Gene Mutations in Familial Ventricular Arrhythmias Resembling Catecholaminergic Polymorphic Ventricular Tachycardia” by Annukka Marjamaa et al. could be reconsidered for publication in the *BMC Medical Genetics*, provided an adequate revision can be carried out. We have very carefully revised the manuscript according to the comments and questions raised by the four Reviewers, and we feel that our manuscript has significantly improved upon the revision.

We thank the Editors and Reviewers for their careful work and hope that our manuscript could be published in its present revised form in *BMC Medical Genetics*.

On behalf of the authors,

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1. Reviewer's report

Title: Search for Cardiac Calcium Cycling Gene Mutations in Familial Ventricular Arrhythmias Resembling Catecholaminergic Polymorphic Ventricular Tachycardia

Version: 1 Date: 28 July 2008
Reviewer: Martin Farr

Reviewer's report:

Major Compulsory Revisions:

The description of the index patients' families (genotypes/phenotypes) remains unclear. It would help to depict the family trees, even if there are no other genetically positive relatives. What is "The rest of the family" of the R1051P index patient - are the parents included, are they genetically negative?! In this paragraph you have to guess that it is concerned with the R1051P mutation until you read it at the end ...

Response:

We thank the reviewers for the interest in our manuscript. We have constructed the family trees of the three patients carrying RyR2 mutations (Figure 3). The Figure 3 also clarifies the relationships of the remaining 18 individuals in the RyR2 R1051P family that were clinically evaluated but did not feature a disease-phenotype nor carried the RyR2 R1051P mutation. The mother of the index patient did not carry the mutation nor showed arrhythmias in the clinical evaluation, the father was not available for the study. We have also clarified the description of RyR2 R1051P phenotypes as follows “The index patient carrying the RyR2 R1051P mutation was 35 years old and had experienced several syncopal spells since the age of 30 (page 11, lines 2-3)... and ”The son of the patient carrying the RyR2 R1051P mutation had a similar phenotype (page 11, line 6)."

For scientific reasons the paternity of the S616L patient's father should be verified. This is important to substantiate the statement 'de novo mutation'.

Response:

We agree with the Reviewer 1 that the definitive statement of the de novo mutation required testing of the paternity of the father. However, we feel that the scientific yield of this information compared to the ethical aspects does not qualify us to such testing. Therefore, we have cautiously interpreted the data as follows: “The mutation appeared to arise de novo as the parents of the index featured no pathological alterations in their exercise stress tests nor carried the RyR2 S616L” (page 11, lines 18-20).

A 'limitation of study' should be added as the number of patients is not high enough to detect mutations less frequent than the RyR2 mutation (only 2 index patients represent 14 percent). Why did the authors expect mutations in the genes tested as/more frequent than in the RyR2?

Response:

The screened candidate genes code for integral proteins in the cardiac Ca^{2+} signalling and thus are closely related to the RyR2. This is the rationale for the study. CPVT and related disorders are rare, and we feel that the present study population of 33 families is acceptable, although limited for definitive conclusions. We have added this limitation of the study to the Discussion (page 16, lines 19-21).

Level of interest: An article whose findings are important to those with closely related research interests.
2. Reviewer's report
Title: Search for Cardiac Calcium Cycling Gene Mutations in Familial Ventricular Arrhythmias Resembling Catecholaminergic Polymorphic Ventricular Tachycardia

Version: 1 Date: 31 July 2008
Reviewer: Zahurul A. Bhuiyan

Reviewer's report:
Authors have searched for mutations in the genes (RyR2, FKBP1B, ATP2A2, SLC8A1) presumed to be involved in CPVT pathogenesis in two groups of CPVT patients (confirmed and likely/doubtful). First group comprised 16 patients with typical CPVT features, where authors have found two mutations in RyR2. Second group comprises 17 patients who were reported to have only VPCs, where no mutation has been detected. Authors have not screened one important gene CASQ2, till now reported pathogenic mutations in CASQ2 are homozygous or compound heterozygous, perhaps this precluded them not to screen the CASQ2.

Overall, it’s a nicely written manuscript.
Several concerns:
A) Authors have included all the coding exons in RyR2. I would suggest to screen the negative patients for the exon-3 deletion, which is not an arduous task. A long segment PCR could easily be done to delineate presence/absence of this deletion in the RyR2 negative cohorts.

Response:
We screened the negative patients for the previously reported 1.1 kb RyR2 exon-3 deletion using MLPA analysis of RyR2 exon 3 (Salsa MLPA Kit P168, MRC Holland, Amsterdam, the Netherlands), with PCR using primers CACAGAACAGGACCAAGTTAGAGG (forward) in intron 2 and CATTACCTTCTGACACACTTCAT (reverse) in intron 3 [1], as suggested by the Reviewer. The RyR2 exon-3 deletion was identified independently in two (13%) CPVT families. We have completely revised the manuscript in this respect, i.e. Abstract, Methods, Results, Discussion and Conclusion. We feel that this finding in conjunction with the existing evidence of the RyR2 deletion is of importance in directing the future studies on RyR2-mediated arrhythmia disorders.

B) I would consider it not logical to say septal hypertrophy linked to RyR2 mutation. This has been compared with the extended phenotypes described by Bhuiyan et al (2007). In fact, none of the RyR2 negative carriers reported by Bhuiyan et al. (2007) had any extended phenotypes. In this manuscript by Marjamaa et al., non-RyR2 carriers also have septal hypertrophy, which suggests this phenotype independent of RyR2 mutation.

Response:
We agree with the Reviewer that the occurrence of cardiac hypertrophy in a distant relative of the two RyR2 R1051P mutation carriers precludes any definitive statements regarding the causative role of the RyR2 R1051P in the mild septal hypertrophy of the index patient. Therefore we have removed this paragraph from the Discussion.

C) Authors have mentioned that, N3308 in RyR2 is conserved, which is not true. This is not conserved between hRyR1 and hRyR3, not also with the RyR2 from other species. This should be modified.

Response:
We have corrected this statement on page 11, line 22 and also added the conservation profiles of the three RyR2 amino acid alterations to the Supplementary Data for clarity.

D) Why only 13% of the patients have a mutation, though previous reports (which includes reports from present authors) detected 40% mutation in RyR2. Was there any selection bias in recruiting patients?

Response:
The 13% (25% in the revised manuscript due to the identified RyR2 exon 3 deletions) frequency of detected RyR2 mutations in the CPVT patients was unexpected. We have very carefully followed the study protocol and are convinced that the patients recruited to the study represent exercise-induced polymorphic ventricular tachycardia similar to our previous studies on CPVT. We believe that our study reflects the existence of either non-coding mutations in the RyR2 gene or genetic heterogeneity of the disorder as discussed on page 14, lines 21-24.

Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: I declare that I have no competing interests

3. Reviewer's report
Title: Search for Cardiac Calcium Cycling Gene Mutations in Familial Ventricular Arrhythmias Resembling Catecholaminergic Polymorphic Ventricular Tachycardia
Version: 1 Date: 12 August 2008
Reviewer: Christopher H George
Reviewer's report:
This is straightforward study of two small cohorts of patients presenting with CPVT and CPVT-like clinical symptoms. There are numerous mutations in the Ca2+-handling proteins RyR2 and calsequestrin that have been linked to CPVT (and other cardiac pathologies that mimic CPVT), although there is a significant incidence of CPVT in the apparent absence of abnormalities in these proteins. The present study identified two novel CPVT-linked mutations in RyR2 (from a cohort of 16) and one apparent polymorphism in a CPVT-like cohort. Logically, the authors attempted to identify mutations in other Ca2+-associated proteins (FKBP, SERCA, NCX1) to explain a candidate molecular basis for their clinical observations. Unfortunately, this proved a fruitless task. Accordingly, apart from the discovery of two novel mutations, the study contributes little new to the field. The absence of additional information as to precisely what underscores the clinical phenotype in the absence of RyR2 mutations (and the presumed lack of mutations in CSQ) leaves the study with a disappointing conclusion.
Major compulsory revisions.
The data on what appears to be a functionally normal sequence variant (N3308S) is too preliminary and does nothing to convince that this variant is in any way connected to the disease phenotype. It is misleading, at this early stage, to state that N3308S is an "RyR2 variant that might play a role in the modification of the phenotype".

Response:
We have deleted this sentence from the Discussion (page 16, lines 1-2).

Why have the authors not investigated the in vitro characteristics of the novel R1051P and S616L mutations that DO appear to be linked with CPVT? This should be done. The authors
comments that the mutational loci is not typical of CPVT (i.e. not found in 'hot-spots'). This makes their lack of functional characterisation even more puzzling.

Response:
We did not perform the single channel recordings for RyR2 R1051P and S616L because 1) both mutations underlie typical CPVT phenotype, 2) they are located in a highly conserved regions of the RyR2 gene, 3) the R1051P fully co-segregates in the family and 4) the mutation loci of S616L in exon 19 resides close to the exon 15 where disease-causing mutations have previously been reported. These new mutational loci most likely represent extensions to the known mutational hot spot regions. We feel that this data is sufficient to declare that these mutations are causative mutations for the disease phenotypes even without supporting \textit{in vitro} data.

It is very surprising that the authors did not investigate CSQ. From other studies, CSQ is robustly associated with CPVT phenotype and in their previous work they identified polymorphisms in CSQ (EJHG 2003). Given that the present work partly focusses on a benign RyR2 variant, the lack of knowledge regarding the existence of CSQ sequence polymorphisms in these same cohorts is a notable omission.

Response:
Our previous screening for mutations in \textit{CASQ2} gene among CPVT patients revealed two common polymorphisms, T66A and V76M, that showed similar allele frequencies among CPVT patients and controls [2]. In the present study, we aimed at searching for rare variants that are more likely to have a causative role for the disease phenotype. In addition, the pedigrees of the present study that showed familial inheritance all supported the autosomal dominant inheritance of the disease phenotype. Since the pathogenic \textit{CASQ2} gene mutations associate primarily with autosomal recessive CPVT, we feel that the exclusion of \textit{CASQ2} gene is justifiable. We have added a note to the Methods (page 7, lines 13-18) for clarity.

Minor essential revisions
Figure 1 is poorly explained in the text.

Response:
We have added an explanation of the Figure 1 (Figure 2 in the revised manuscript) in the Results section (page 10, lines 8-10).

Page 9, paragraph 2 is rather confusing. For R1051P, the index patient's son has a similar phenotype but did he also carry the mutation?

Response:
We have clarified this section in the Results (page 11, line 6) and also depicted the family trees (Figure 3) as recommended by the Reviewer 1.

The single channel data should be labelled as Figure 2

Response:
We have corrected the labelling of the single channel data (page 13, line 12, Figure 4 in the revised manuscript).

p12. Although the work of Bhuiyan is cited, the authors should refer to recent data in which RyR2 mutations have been linked to a longQT-like phenotype.

Response:
We have completely rephrased the Discussion in this respect.
p4, para 2. The authors should acknowledge the controversy surrounding the RYR2:FKBP12.6 interaction in heart disease

Response:
We have added a phrase acknowledging this controversy to the Background (page 4, lines 13-14).

p7 para 2. Is the full-length co-expressed with FKBP12. HEK cells already express abundant FKBP12 so should this read FKBP12.6?

Response:
We have corrected the labelling on page 8, line 6.

Level of interest: An article of limited interest
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests: I declare that I have no competing interests

4. Reviewer's report
Title: Search for Cardiac Calcium Cycling Gene Mutations in Familial Ventricular Arrhythmias Resembling Catecholaminergic Polymorphic Ventricular Tachycardia
Version: 1 Date: 19 August 2008
Reviewer: Jonathan G Seidman
Reviewer's report:
The authors identified 33 consecutive patients with frequent ventricular premature complexes (VPCs) without structural heart disease and frequent history of syncope or sudden death in family. 16 of 33 patients featured exercise induced VPCs, while the other 17 showed VPCs at rest. The RyR2, FKBP1B, ATP2A3 and SLC8A1 genes were sequenced in all 33 patients. 2 novel CPVT-causing RyR2 mutations were found among 16 subjects in the exercise induced VPC group and a novel RyR2 variant of uncertain clinical significance in a patient with abundant resting VPCs. The mutant N3308S variant RyR2 was tested in vitro.

Major comments:
1) The clinical criteria used to define CPVT is unclear in this manuscript. Do both groups A and B have CPVT? Or Only group A has CPVT? In any event, examples of polymorphic beats on the ECG should be presented. Given that RyR2 variants are found in members of both groups, there is reasonable evidence that the two groups are not particularly different. Perhaps the frequency of sequence variants in RyR2 should be 3 in 33.

Response:
We used the following criteria in recruiting the patients to the study: 1) patients had frequent ventricular premature complexes (VPCs) during exercise 2) and consecutive VPCs, history of syncope and/or sudden juvenile death in family. None had apparent structural heart disease or prolonged QT interval. As CPVT is defined as exercise-induced ventricular tachycardia, we consider the group B with abundant VPCs also in resting conditions to present a phenotype different from the typical CPVT and feel that it should be evaluated separately. We have constructed a figure showing examples of the exercise stress test from both groups for further clarity as suggested by the Reviewer (Figure 1).

2) The nucleotide sequence traces of the three mutations should be presented as supplemental data.

Response
We have constructed a figure showing the forward and reverse sequence traces of the three RyR2 missense mutations (Supplementary Data).

3) The pedigrees of the three families with RyR2 sequence variants should be presented. The pedigrees should indicate the clinical diagnosis and genotype of family members. This can be either supplemental material or placed in the text.

Response:
We have depicted the pedigrees of the three families and present their phenotype-genotype correlations (Figure 3).

4) Sequence variants S616L and R1051P should also be tested in vitro.

Response:
We did not perform single channel recordings of RyR2 R1051P and S616L because both highly conserved mutations underlie typical CPVT phenotype. Since R1051P co-segregates with the disease phenotype in the family, and since S616L resides in exon 19 close to the exon 15 where disease-causing mutations have previously been reported, we feel that this data is sufficient to declare that these mutation present causative mutations even without supporting in vitro data.

Minor comments;
In table1 and figure 1 legend: Presumably, bmp should be “bpm”.

Response:
We have corrected the typing error in Table 1 and in the Figure 1 Legend (Figure 2 in the revised manuscript).

In table1, the QTc range is up to 470ms. Is this correct? A patient with a QTc of 470ms has a prolonged QT interval; this prolonged QT interval may be responsible for TdP.

Response:
We have checked the ECG of the study subject and the correct QTc interval is 450 ms. Considering the internationally accepted threshold value of 460ms for prolonged QTc interval in females [3], we consider this QT interval to be within normal range. No TdPs have been documented in the study subject.

Level of interest: An article of importance in its field
Quality of written English: Needs some language corrections before being published
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests.