Author's response to reviews

**Title:** Exome sequencing helped the fine diagnosis of two siblings afflicted with atypical Timothy syndrome (TS2)

**Authors:**

Sebastian Fröhler (Sebastian.Froehler@mdc-berlin.de)
Moritz Kieslich (Moritz.Kieslich@charite.de)
Claudia Langnick (Claudia.Langnick@mdc-berlin.de)
Mirjam Feldkamp (Mirjam.Feldkamp@mdc-berlin.de)
Bernd Opgen-Rhein (Bernd.Opgen-Rhein@charite.de)
Felix Berger (Felix.Berger@charite.de)
Joachim C Will (Achim.Will@charite.de)
Wei Chen (Wei.Chen@mdc-berlin.de)

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**Author's response to reviews:** see over
Referee 1 - Jill Johnsen

Reviewer's report:
Fröhler et al describe a tiered approach to identifying the causative mutation in a family with arrhythmia, first by a candidate gene approach, and then utilizing whole exomes when no clear mutations were found in the candidate genes. The pedigree exhibits early childhood onset long QT syndrome (LQTS) in two siblings. Through whole exome sequencing they identified a p.G402S CACNA1C gene variant previously implicated in Timothy syndrome, a syndrome which includes cardiac arrhythmias and for which there is experimental evidence as to the function of variants at this residue. The authors conclude that a genome wide approach was effective and should be considered in the pursuit of the molecular diagnosis of suspected inherited syndromes.

Major Compulsory Revisions:

Revision 1: Manifestations of the syndrome (partial syndactyly) should be included earlier in the description of the cases (perhaps a table of characteristics?). There needs to be clarification if it has been shown that the other manifestations of the syndrome are truly absent, rather than not overtly present but not investigated?

Answer: Both children show prolonged QTc interval and partial syndactyly. Syndactyly is only seen in TS type 1. Both children do not have cranio-facial dysmorphies and do not suffer from recurrent infections (as seen in TS type 1+2). CNS features in Patient 1 can be interpreted as neurological sequelae after resuscitation (see below). Both children have been seen by pediatricians and pediatric neurologist. Accordant tests (e.g. EEGs) for features of TS have been performed.

Clinical features are now described more clearly and early in the manuscript, a table summarizing clinical features of Timothy Syndrome in our patients has been added (Table 1). The following text has been added to the manuscript:

"After receiving the molecular diagnosis, mild partial syndactyly of the second/third toe, which usually is regarded as a normal variant, was found in both patients as well as in their father. It is speculative whether this finding could be related to the p.402G>S mutation, since the few reported patients carrying this mutation do not show syndactyly [9]. Both children do not meet the full clinical criteria for classical TS apart from the prolonged QTc interval: Their hearts are structurally and functionally normal, they do not show any cranio-facial dysmorphies, they do not suffer from recurrent infections and no other major anomalies as manifested in TS (type 1) can be detected (see Additional file 1)."

Revision 2: Please comment if the cognitive/developmental delay in the proband could be entirely attributed to the brain injury acquired at the time of her first event and resuscitation, or could that traumatic/anoxic event be confounding clinical detection of developmental delays truly consistent with the syndrome?

Answer: The features are more likely a sequel of the hypoxic event than truly part of the syndrome because 1) the proband’s development was unremarkable prior to the event; 2) in the brother (who is carrying the same mutation) no CNS abnormalities could be detected. It is not possible to make a definite conclusion. Nevertheless, the pediatric neurologists prefer to attribute the cognitive/developmental delay and epilepsy to hypoxic brain injury (consistent with imaging and tests performed after the event).

The following text has been added to the manuscript:

"The neurological findings (epilepsy, developmental delay) are rather attributed to hypoxic brain damage caused by cardiac arrest and resuscitation than being part of
TS. Especially since the early neonatal development of the patient has been entirely unremarkable. Moreover, patient 2 unlike his sister reached the milestones of psychomotor development at the expected times and clinical workup failed to show any neurological abnormalities. He is the second child of the family and is now six years old.”

Revision 3: Has the father had an EKG or other investigations?

Answer: The father's EKG is unremarkable (normal Qtc interval).

The following text has been added to the manuscript:

“Electrocardiograms of both the father (who was found to be a p.G402S mosaic variant carrier) and the mother are unremarkable and show a normal QTc-interval. The father’s 6 siblings who live in Lebanon were not investigated (no ECGs).”

Revision 4: The authors make the case that the father is relatively unaffected, and attribute this to mosaicism, which is entirely plausible, based upon the relative number of reads in the next-gen data with the mutation, the chromatogram peak height of the variant in the Sanger sequencing, and the relative absence (?) of smaller bands using a restriction digest specific to the variant. However, there are still other explanations for these findings, such as structural variants such as CNV or artifacts. Confirmation on an independent DNA sample would be warranted if it has not been done, and consideration of sequencing DNA from another tissue might offer stronger evidence of mosaicism.

Answer: A second validation of the mutation in father, son and daughter has been performed on oral mucosa swap samples obtained from each individual, confirming the mosaic variant at a similar low allele frequency in the father, as well as a heterozygous genotype for both children. Sanger-traces of this second validation is available as Supplementary Figure 1 (Additional file 3).

A modified statement was added to the text:

“Sanger sequencing on the blood samples of all family members not only confirmed the heterozygous p.402G>S variant in both affected children but also the mosaic genotype for this variant in the father (Figure 1b). The father's mosaic genotype was also observed in his oral mucosa swap sample at a similar allele frequency (see Additional file 3).”

Revision 5: Page 10, the authors state, ‘So the less sensitive method, in this case, appeared to be more sensitive?. This needs to be revised, the methods are different, these finds are not truly an approach to make comments on the 'sensitivity' of the methods.

Answer: It has been reported before that GATK and samtools, due to similar but partly different methodology, show a very high but incomplete overlap w.r.t variants called (Lam et al., PMID: 22398614, O’Rawe PMID: 23537139). Based on the data from Lam et al. and O’Rawe et al., GATK appears to be slightly more sensitive, when compared to samtools. This is also what we observed in our study. However, with such a single case, we agree that this manuscript is no systematic assessment of the performance of variant callers. The sentence in question has been removed from the manuscript.

Revision 6: Was the informed consent that was signed under an IRB or similar?

Answer: Written informed consent was obtained for this clinical molecular diagnostics from each patient.
The following text has been added to the manuscript:

“Written informed consent was obtained from each family member for the subsequent studies according to the German Genetic Diagnostic Law (GenDG).”

**Discretionary Revisions:**

**Discretionary Revision 1:** The structure of the article is narrative, but would be improved by at least loose organization such that the methods, results, and discussion together.

**Answer:** Due to the article structure suggested by BMC Medical Genetics, we feel bound to the current separation of our manuscript into Methods, Results and Discussion.

**Discretionary Revision 2:** What is the explanation for the precocious puberty in the proband? Is this suspected to be part of the syndrome?

**Answer:** Precocious puberty has not been described to be part of TS 1+2.

The following text has been added to the manuscript:

“She” ... “additionally shows evidence of a vast precocious puberty which is not known to be associated with TS. The neurological findings (epilepsy, developmental delay) are rather attributed to hypoxic brain damage caused by cardiac arrest and resuscitation than being part of TS. Especially since the early neonatal development of the patient has been entirely unremarkable. Moreover, patient 2 unlike his sister reached the milestones of psychomotor development at the expected times and clinical workup failed to show any neurological abnormalities.”

**Discretionary Revision 3:** What about the father’s relatives? Although seemingly unlikely, mosaicism has multiple proposed origins that could lead to risk for a relative, particularly a sibling.

**Answer:** The following text has been added to the manuscript:

“The father’s 6 siblings who live in Lebanon were not investigated (no ECGs).”

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**Reviewer 2 - Jacob Tfelt-Hansen**

Reviewer's report:
This is a potentially interesting paper on a genetic work up on two LQTS syndrome children with unrelated parents and with a severe phenotype. The siblings were genonegative for the 5 most common LQTS genes and the authors used an approach of NGS. They report the finding of a G402S mutation in exon 8 of the CACNA1C gene. The father who is asymptomatic is reported to be mosaic variant carrier. Based on the gene finding they change the diagnosis from LQTS to Timothy syndrome.

**Major limitations:**
**Revision 1: Did the siblings fulfil the Timothy syndrome criteria?**

**Answer:** No, they did not manifest the full clinical spectrum of TS2 (see table of characteristics, newly added). Both children show prolonged QTc interval and partial syndactyly. Syndactyly is only seen in TS type 1. Both children do not have cranio-facial dysmorphies and do not suffer from recurrent infections (as seen in TS type 1+2). CNS features in Patient 1 can be in interpreted as neurological sequelae after resuscitation. Please see also reply to reviewer 1 on the same question.

**Revision 2: The NGS panel should be listed**

**Answer:** The set of gene regions investigated during exome sequencing comprise all regions covered by the commercially available ‘Agilent SureSelect V4’ exome enrichment system (33,805,644 exonic bases of 21,602 UCSC hg19 RefSeq genes). A list of screened genes and a list of assayed regions for these probes is available on the Agilent website (https://earray.chem.agilent.com/suredesign/).

The following text has been added to the manuscript:

“A list of screened genes and a list of assayed regions for SureSelect V4 probes is available on the Agilent website (https://earray.chem.agilent.com/suredesign/).”

**Revision 3: The use of the genetic finding to change the diagnose should be discussed with pro and cons**

**Answer:** Exome sequencing, as performed in this study, offers an unbiased approach to unravel the molecular causes of Mendelian disorders. A clear advantage of this next-generation-sequencing (NGS) based strategy is the comprehensive screening of (almost) all coding bases in the patient's genome, compared to only a very small set of target regions, e.g. in the LQT gene panel test that was performed initially. This is especially relevant for rare diseases. With today’s technology, such unbiased screening can be achieved at a comparable or even lower price when compared to single-amplicon (Sanger) based tests. This allows for a more appropriate genetic counseling. Nevertheless, for the application of such technology in clinical context, data protection and anonymization are key issues which should be thoroughly controlled by legal authorities and respective laws (e.g., the Genetic Diagnostic Law in Germany).

As a ‘side-effect’ of an unbiased mutation profiling, additional molecular findings, not related to the disease to be studied initially, may appear, which need to be assessed very carefully by a specially trained genetic counselor. The right of the patient ‘not to know’ those incidental findings must be respected under all circumstances and must be queried when obtaining informed consent for a study. Furthermore, attributing a patient to a 'syndrome' can stigmatize the patient. Finally, it needs to be addressed by federal ethics committees whether molecular diagnosis with unclear relevance should be conveyed to the patient at all or if only molecular findings with clear relations to a disease must be reported.

The following text has been modified and added to the manuscript:

“Combined with a genotype-phenotype-treatment resource, constantly expanding during such kind of studies in the clinic, new therapeutic approaches could be offered to the patient as an immediate benefit. However, in such clinical context, controlled vocabularies for phenotyping patients together with rigid data protection strategies must be warranted at all times. Additional legislative effort
might be required for recording, storing and accessing this kind of data. Furthermore, a clear consent must be obtained regarding how to proceed with incidental findings. Whether those should be conveyed to the patient if requested, whether to only convey results on 'treatable diseases' or even only to convey findings immediately relevant for the disease studied must be agreed upon before initiating such kind of studies. Finally, diagnosing a 'syndrome' might stigmatize the patient, so proper genetic counseling by specialized physicians will be required for all such molecular genetic diagnostics."

Minor limitations:

**Discretionary Revision 1: The differences between the two methods should be explained in depth.**

**Answer:** It has been reported before that GATK and samtools, due to partly different methodology, show a very high but incomplete overlap w.r.t variants called (Lam et al., PMID: 22398614, O'Rawe PMID: 23537139). Based on the data from Lam et al. and O'Rawe et al., GATK appears to be slightly more sensitive, when compared to samtools. This is also what we observed in our study. However, with such a single case, this manuscript is no systematic assessment of the performance of variant callers. Therefore we feel there is no necessity to discuss the methodology in depth.

**Discretionary Revision 2: It should be stated if the mutation is found in EVS and 1000 genome project?**

**Answer:** As of 02/12/2013, neither the 1000genomes phase1 database, available at [http://www.1000genomes.org/](http://www.1000genomes.org/), nor the EVS database, available at [http://evs.gs.washington.edu/EVS/](http://evs.gs.washington.edu/EVS/), does list any mutations overlapping the 1bp interval (BED-coordinate, UCSC hg19: chr12:2613691-2613692) of the SNV we describe in our manuscript.