Author’s response to reviews

Title: Functional study of DAND5 variant in patients with Congenital Heart Disease and laterality defects

Authors:

Fernando Cristo (fernando.cristo@nms.unl.pt)
Jose Inácio (jose.inacio@nms.unl.pt)
Salomé Almeida (salome.almeida@chlc.min-saude.pt)
Patricia Mendes (patimendes@gmail.com)
Duarte Saraiva Martins (duartesaraivamartins@gmail.com)
José Maio (josefmaio@sapo.pt)
Rui Anjos (ranjos@netcabo.pt)
José Belo (jose.belo@nms.unl.pt)

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Manuscript (MGTC-D-16-00031R1) - Response to reviewers.

Cristo el al, 2016

Here, as you may appreciate and as you point out in your decision letter, during the first submission of the manuscript by Cristo el al, 2016, we conducted and added new experiments to addressed all the reviewer’s comments:

Reply to the Reviewers:

Reviewer 1 (Alex Postma) accepted the manuscript without any further revision.

Reviewer 2 (Georges Nemer) addressed the following questions:

1- The inheritance of the variant from the healthy mother in one family coupled to the lack of data from the other argues against a role of this variant in the disease.
Several studies demonstrate that there are an enormous variability in expressivity and penetrance of variants related to CHD. Here, we point that DAND5 is an allele that most probably contributes as part of a complex traits to CHD because the results did not support a specific phenotype-genotype link. This was addressed and clarified in the text.

2-
   a- The authors cannot add the different genes altogether and not present the individual effect for each one of them

   b- A dose response should be included for the mutant protein versus the wild type protein

   c- A western blot should be included to assay equal expression for both proteins

As mentioned before, we performed the luciferase assay according to the work published by Mohapatra et al., 2009 and more recently, Inacio et al., 2013, which is a standard, robust and reliable proceeding to test Nodal signaling activity.

Nevertheless, in this new version of the manuscript we performed a new luciferase assay adding the individual effect for each gene present in the assay as asked by Reviewer 2. In addition, we complemented the assay with a dose response for the mutant protein versus the wild type protein. The results even more confirmed that increasing amounts of DAND5 have increasing levels of inhibition of NODAL signaling.

Regarding the western blot, to demonstrate the equal expression of DAND5 WT and DAND5 variant, we have now tried two different antibodies against human DAND5 (N-terminal (ref ab136327) and C-terminal (ref ab136293)) using several WB protocol conditions but, unfortunately, we did not detect any signal for both wild-type and variant proteins. The other commercial available DAND5 antibodies were raised against the same aminoacid sequence as the ones we tested, therefore, for our disappointment, it was not possible to address this question using the human proteins. However, as the human and the mouse proteins share a significant identity and similarity, 60.7% and 65.96%, respectively, being even basically identical (98%?) within the sequence region containing the variant defined between the 2 Cystein residues (see Figure 1B), we performed a new western blot using lysates of cells transfected with the mouse DAND5/Cerl2 WT and DAND5/Cerl2 variant proteins, cloned in the same expression vector used for the human luciferase assay, and no difference was observed in the protein production in the lisa. Curiously, the conditioned medium of the same cells showed a higher accumulation of the DAND5/Cerl2 variant protein when compared to the DAND5/Cerl2 WT protein. This result might suggest that the aminoacid alteration on the DAND5 protein may be even more deleterious then we would concluded form the luciferase assay once that being apparently more secreted protein than the Wt and even so, lower inhibitory activity. Both the experiments using either the human or the mouse proteins were repeated 3 times.

Reviewer 3 (Denise Kay) raised the following concerns:

1. The frequency of the p.R152H variant in patients (0.026) is essentially the same as it is in the ExAC European control population (0.024). Without a formal test for association using controls
derived from the same population as patients, it can only be suggested (rather than concluded) that the variant is a risk allele.

We agree with the Reviewer 3 and the text was modified as suggested.

2. Table S1 should be included as one of the 'main' tables, not as supplementary material.

Table S1 was included as a main table as suggested by Reviewer 3.

3. In the current study, 2/38 individuals were heterozygous carriers of p.R152H. Table S1 currently only shows the number of ExAC subjects homozygous for this variant. The number of heterozygous carriers of this variant in each ExAC population should be added to Table S1.

Table S1 was modified as suggested by Reviewer 3.

4. Please add patient ascertainment to methods. Were all subjects ascertained from tertiary/referral clinics in Portugal?

Yes, paediatric cardiologists (Dr. Anjos and Dr. Maio) are following all patients. Patient ascertainment was added to methods.

5. The authors should state in the methods that screening known CHD / laterality genes was not conducted.

The text was modified as suggested by the reviewer.

6. Even if age-matched controls aren't available, the variant should be screened in race/ethnicity- and geography-matched controls. If this is not possible, that the variant was not screened in controls derived from the same population as their patients (Portugal, I assume, please see above) should be mentioned in the discussion as either (1) a weakness of the study, (2) an area for future research, or (3) both.

The ethnicity- and geography-matched controls were inserted and the text modified accordingly.

7. The authors response regarding dominant vs. recessive effect (including mention that all DAND5 heterozygous mice are phenotypically normal) should be added to the paper.

The text was modified as suggested by the reviewer.

8. Please add a sentence to indicate no other DAND5 mutations or polymorphisms were detected in the 38 individuals (per the ExAC database, p.R152H is the most common variant [detected by WES] in DAND5).

The sentence was added as suggested.
9. I suggest rephrasing lines 293-295 (pg. 12) as follows: ‘...in all populations but never reported to be associated with disease. The variant has been detected in the heterozygous state in more than 1,200 individuals in the ExAC dataset, and is even present in 16 apparently normal homozygotes.’

Modified as suggested.

10. Line 304: Please comment on the frequency of heterozygotes in ExAC, compared to the expected frequency of undetected CHD / laterality defects or cases with unreported CHD / laterality defects in the ExAC dataset.

Corrected as suggested by the reviewer