Author's response to reviews

Title: Sequencing of NOTCH1, GATA5, TGFBR1 AND TGFBR2 genes in familial cases of Bicuspid Aortic Valve

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Author's response to reviews: see over
Editors of “BMC Medical Genetics”

Re: MS: 1086602316838228

Title: Sequencing of NOTCH1, GATA5, TGFBR1 and TGFBR2 genes in familial cases of Bicuspid Aortic Valve

Dear Editors,

we are grateful to Reviewers for useful and constructive suggestions. In the new version we tried to resolve all the issues raised by reviewers and answer accordingly.

The added sections in the manuscript according to the Reviewer’ comment are indicated in red bold italic. We hope that the revised manuscript adequately addressed all criticisms.

The authors state that the manuscript has not been published and is not being considered for publication elsewhere in whole or in part in any language.

The authors disclose any financial associations that might pose a conflict of interest in connection with the submitted article. All co-authors have read and approved the submission of the manuscript to BMC Medical Genetics.

Yours sincerely,

Dr. Ilenia Foffa
Referee 1

Major points:
The paper background should be expanded, as the cases described, BAV and CoA, represent part of the spectrum of left ventricular outflow tract malformations that include congenital aortic valve stenosis and hypoplastic left heart syndrome. NOTCH1 mutations have been noted in individuals with those phenotypes by our group (McBride et al Hum Mol Genet. 2008; Riley, McBride, Cole Biochem Biophys Acta 2011) and others (Iascone et al Clin Genet 2012), and were also seen in the original report by Garg et al. There is some suggestion that NOTCH1 mutations may contribute to calcific aortic valve disease, but may play less of a role in BAV with aortic dilation (Kent et al J Thorac Cardiovasc Surg 2012). GATA5 has recently been implicated in both mouse and human studies as a cause of BAV, but has not been described in other LVOT malformations.

We thank the reviewer for the comments. We expanded the background as suggested:
Interestingly, McBride et al., demonstrated that mutations in the gene NOTCH1, are found in cases with in cases of left ventricular outflow tract (LVOT) malformations including aortic valve stenosis, coarctation of the aorta and hypoplastic left heart syndrome [13]. In particular, a much higher rate of bicuspid aortic valve (BAV) has been found in families with LVOT malformations compared to the general population, suggesting that BAV may be a forme frusta of the more serious LVOT malformations [13].

Conversely from NOTCH1 gene, the involvement of GATA5 has not been described in other LVOT malformations.

Methods: It is unclear in the methods section if the families include 2 or 3 total affected individuals. How was the diagnosis of BAV confirmed in each family member? I am not certain what a presumptive diagnosis of BAV might mean.

Your observation is right. We corrected the sentence:
Familial BAV was defined if 2 or more affected relative had proven BAV as diagnosed by echocardiography or cardiac magnetic resonance.

Results: Results: The score or specific result from the PolyPhen2 or SIFT analysis should be stated.

We modified Results section as suggested:
PolyPhen-2 analysis predicted the P284L mutation like probably damaging with a score of 0.993. This result was confirmed by SIFT (score:0.04).
The missense mutation (P284L) identified in our proband, tested by MutationTaster, induced the lost of an important residue with putative significance for calcium binding to epidermal growth factor (EGF)-like 7 domain.

And we better described PolyPhen-2 and SIFT tools in the method section:
PolyPhen-2 predicts the effect of an amino acid substitution on the structure and function of a protein using sequence homology. The PolyPhen-2 score represents the probability that a substitution is damaging, so values nearer 1 are more confidently predicted to be deleterious (note that this the opposite to SIFT).
SIFT uses sequence homology to predict whether an amino acid substitution will affect the protein function. SIFT score <0.05 indicates the amino acid substitution is damaging while a scores ≥ 0.05 are predicted to be tolerant
It would be helpful to indicate the phenotypes on the pedigree figure for each individual.

*Your observation is right we added more information about the phenotype of each family member in table 1. In the text we added:*

Patient’s phenotype is reported in table 1. Two of the probands (18%) had BAV with unknown morphology. Of the 9 probands with known BAV morphology, 7 (63%) had fusion of the right/left coronary commissure whereas 2 (18%) had a valve with anterior-posterior sinus without raphe.

The authors mix allele frequency and genotype frequency when commenting on the GATA5 variant rs6142775 (p.Thr67Pro): the allele frequency is 0.22 in their cohort. They do not state what the frequency of this variant was in their own control group. The frequency of this variant is in fact present in dbSNP, where data from the 1000 Genomes Project shows a minor allele frequency of 0.15 in Europeans and 0.17 in the Toscani Italian subpopulation. Thus this variant is NOT a very rare allele, but a common polymorphism.

*We modified the text following the recommendation:*

Of note, in 5 patients (45%) we found the Thr67Pro non-synonymous variant, previously described by Padang et al in BAV patients, that is a common polymorphism in European population with an allele frequency of 0.15.

Discussion: I agree with the findings of the NOTCH1 mutations they found, but more information is required on the bioinformatics analyses they performed.

*We added more information about bioinformatics analysis performed (see method and result sections. Moreover we used MutationTaster a free, web-based application for a rapid evaluation of disease-causing potential of DNA sequence alterations.*

I disagree that NOTCH1 mutations are responsible for a large percentage of BAV cases; their study is very small, and when combined with other studies suggest a role for NOTCH1 mutations in a few percent of individuals with BAV.

*We modified this part of discussion as suggested:*

Our findings confirm the important role of NOTCH1 as conserved intracellular regulator in the pathogenesis of congenital valve disease and in its complications. Indeed, the finding of two independent de novo mutations in this gene in a small number of affected subjects (11 patients) constitutes compelling evidence of disease causation.

Discussion: I disagree with their discussion of GATA5 regarding the rs6142775 variant. There is no data to back their claim it is a modifier. They have misread the paper by Padang et al; the variant was not reported as being absent in controls, but that it was not assessed in their control group. In addition, the frequency of the variant in their cohort does not appear to be different from other European populations, and they do not provide a frequency of this variant in their own control cohort. The current discussion and conclusions for this variant should be removed, and the variant should be described as simply a variant of no significance.

*Your observation is right. We modified the part of the discussion of GATA5 regarding the rs6142775 variant:*

Conversely, we did not identify any pathogenetic mutations in GATA5. The non-synonymous variant Thr67Pro located in the transcriptional activator domain encoded by exon 1, detected
with a frequency of 45% in our population, is a common genetic polymorphism previously described by Padang et al.

Additional comments:
Please review the paper for proper use of gene and protein names. All human genes should be italicized and all caps, proteins should be all caps and not italicized. Rodent genes are italicized and have the first letter capitalized, with proteins not italicized. The proper abbreviations is TGFBR1 and TGFBR2 not TGFBR1 and II. The attached file contains some track changes for English wording suggestions.

We checked and corrected the errors. Thanks.

Referee 4

- It is mandatory that the nomenclature of mutations follow closely the recommendations of the HGVS (http://www.hgvs.org/mutnomen/). It is crucial to give full info (position on genomic DNA, on cDNA) and to let the reader know which assembly and which databases were used.

The observation is right. We adjusted the nomenclature of mutations and we added more information about the assembly and database used in the tables

-Study population: The authors state that familial BAV was defined when '2 or more affected relatives had presumptive or proven BAV'. This wording is easy to misunderstand. Were any families defined as multiplex families in the absence of imaging data? The word 'presumptive' means 'not proven'.

We better clarify this point in the Study Population as suggested also by referee 1. Please also see the response to referee 1

- The family pedigrees A and I should give the full segregation pattern of the NOTCH mutants. If possible, the authors should extend familial screening by imaging all living first-degree relatives of the affected parent. Were there any other offspring in those families, or miscarriages?

We agree with this comment, but the familial screening of all living first degree relatives was not possible because the probands declared their death. Moreover they have not miscarriages and have only one child.

- The authors need to give a full account of the phenotype of all family members by echocardiography (or other imaging method) and need to depict the allele status for every family member.

Your observation is right. We added more information about the phenotype of each family member in Table 1. In the text we added:

Patient’s phenotype is reported in table 1. Two of the probands (18%) had BAV with unknown morphology. Of the 9 probands with known BAV morphology, 7 (63%) had fusion of the right/left coronary commissure whereas 2 (18%) had a valve with anterior-posterior sinus without raphe.

- Genetic analysis of GATA5: I did not find the Thr67Pro variant in the exome server, as stated by the authors. It is important to note that the frequencies given by the authors for the control
population are heavily skewed through a dataset from the Maslen lab, which seems to have submitted sequencing data from a congenital heart disease dataset. The authors do not give the frequency of this variant in their ethnically matched controls and should absolutely do so. Of great interest, Padang seems to have identified the same variant in their BAV patients.

*We better clarify this point and added in the text:*  
Of note, in 5 patients (45%) we found the Thr67Pro non-synonymous variant, previously described by Padang et al. in BAV patients, that is a common polymorphism in European population with an allele frequency of 0.15.

- the meaning of the word 'strumental” is unknown.  
*We corrected using instrumental analysis*

Referee 5

2) The methods used are standard and appropriately chosen for the study. The only concern is that the PCR amplification is subjected to nucleotide mis-incorporation and the authors should state how many independent PCR replicates have been sequenced, particularly in the cases, where a mutation has been detected. Considering that the main core of this report is identification of the point mutations in several genes and the small number of patients (11), the possibility of nucleotide misincorporation should be excluded, even though they have 200 biological replicates (controls).

*We better clarify this point in the Methods section:*  
Detected mutations were confirmed by sequencing in the opposite direction from other two independent PCR products.

6) I think the authors have to mention that their sample size is very small (11 patients).  
*We agree with the referee and added this point as study limitation in the text.*

Although the small number of patients (11) might be an important study limitation

7) Totally, there are four articles published on *NOTCH1* mutations in patients with BAV, the authors discuss three of them, but not the most recent one (PMID: 23102684). In the most recent paper(PMID: 23102684), the authors summarize the previous data on *NOTCH1* mutation in relation to patients valve calcification and aortic dilation and argue that the existing data does not establish the *NOTCH1* mutations contribution to ascending aortic aneurysms in non-calcified BAVs. According to this view, there are two types of BAVs; (i) the *NOTCH1* dependent BAVs, associated with stenotic, or calcified aortic valves and (ii) the *NOTCH1* independent ones with non-calcified aortic valves. Clearly, the proband P284L mentioned in the present study has had a severely calcified valve, with aneurysm development, a year later. The second patient carrying a mutation in exon 26 has also been through a valve surgery, most probably due to a dysfunctional valve. So their data seems to agree with and fit well into the proposal made in 23102684 article, and this issue should be addressed in their discussion.

*We better discussed this point raised by the reviewer in the new version, quoting the recent paper.*

In a very recent paper, Kent et al. suggested a *NOTCH1*-dependent mechanism that produces stenotic, insufficient and/or calcified aortic valve with rare aneurysm, and a *NOTCH1*-independent mechanism that produces highly penetrant AscAA in the presence of a non-
calcified and often normally functioning BAV(30). Our data seems to agree with and fit well into the proposal made in this article since we found two novel mutations in patients with valve malformation, calcification, and dysfunction as predominant phenotype caused by altered NOTCH1 signaling.