Author’s response to reviews

Title: The impact of down-regulated SK3 expressions on Hirschsprung disease

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RESPONSES TO REVIEWERS AND EDITOR:

In the material below, the reviewer comments are in italics and our responses are in regular type.

Reviewer #1 (Aldamaria Puliti):

- The manuscript deals with the persistence of bowel symptoms after a properly performed pull-through operation, an important point for Hirschsprung disease affected patients.

We appreciate the encouraging comments of this reviewer.

- The Authors analysed by real-time PCR the expression level of SK3 RNA in aganglionic and ganglionic colon regions of 14 HSCR patients compared to the ganglionic colon of 6 controls. Data indicate that in all analysed patients the expression of SK3 is reduced with respect to controls which leaded the Authors to sustain a role of SK3 in the persistent bowel symptoms. To explain these dysmotility problems, it has been previously hypothesised that ganglionic colon, just proximal to the transition zone, could have an anomalous inhibitory neurotransmission, and that SK3 channel expression from PDGFRa+ cells could be implicated. Data in support of this hypothesis have been recently published by Coyle and collaborators (Journal of Pediatric Surgery, 2015). This point deserves much more
investigation, as extending the number of investigated patients. In this view, the manuscript presented by Gunadi and collaborators represents an important work, of interest for pediatric surgery.

We appreciate the encouraging comments of this reviewer.

- However, the Authors do not provide any evidence on a genetic contribution of the SK3 gene on the HSCR development. They do not report on the genetic possible causes of HSCR in the analysed patients (e.g. RET screening). They do not investigate genetic variations that could affect SK3 expression, and might support SK3 as susceptibility gene or to have a direct role in persistence of bowel symptoms. Indeed, the conclusion that SK3 expression could be an important marker for genetic counselling is too speculative.

We have now provided the genotyping data of the RET rs2435357 variant for HSCR patients in the Table 1 since this variant has been strongly associated with HSCR in Indonesia (Gunadi et al., 2014; 2016), and added the following sentences in the Results section: “First, we genotyped HSCR patients for the RET rs2435357 variant since this variant has been strongly associated with HSCR in an Indonesian population [5,6]. The genotype frequencies for RET rs2435357 variant among HSCR patients were as follows: TT (12/14, 86%), CT (0), and CC (2/14, 14%).”

- In fact my great concern is on the subject of the research performed by the Authors which, in my opinion, seems to be not in the "Aims and Scope" of the BMC Medical Genetics Journal, probably of interest for a surgery journal. Indeed, in the present form the manuscript is not acceptable. Hereafter some suggested points that could improve the quality of the manuscript.

We have now revised the manuscript per your helpful comments and hope that we have now produced a more balanced and clearer account of our work.

Major points

- Methods: RT-PCR: please indicate the quantity of RNA used for cDNA synthesis.

We have now revised the Methods section to the following sentences: “The SK3 expression was quantified using 100 ng of total RNA, the Kapa SBYR Fast qRT-PCR One Step Kit Universal (Kapa Biosystems, Massachusetts, USA), and the BioRad CFX Real-Time PCR System (California, USA).”
Methods: RT-PCR: please detail that "……… to determine the SK3 mRNA expression level in ganglionic and aganglionic colon from HSCR patients normalising to GAPDH and relative to ganglionic colon from control individuals."

We have now changed the Methods section to the following sentences: “The Livak (2-ΔΔCT) method was utilized to determine the SK3 mRNA expression level in ganglionic and aganglionic colon from HSCR patients normalizing to GAPDH and relative to ganglionic colon from control individuals.”

- Table 1: please split into two separate columns the number of patients (not %), and age.

We have now revised the Table 1.

- Table 1: if possible, add a column indicating the genotype of the patient (RET/other gene mutation?)

We have now provided the RET rs2435357 genotyping of HSCR patients in the Table 1.

- Table 1: add a column with symptoms present in the patient, if any

We have now added the persistent bowel symptoms in the HSCR patients in the Table 1.

- Table 2: please replace "Mean difference" with ΔΔCt

We have now replaced “Mean difference” with “ΔΔCT” in the Table 2.

- Table 2: please insert a column reporting the fold change

We have now inserted the fold change in the Table 2.

- Table 2: please complete the legend *, p<……… versus control

We have now completed the legend of Table 2 to the following sentence: “*, p<0.05 is considered statistically significant for the SK3 expression difference between HSCR patient versus control colon.”
• Figure: I suggest to modify the figure to better evidence the difference between ganglionic and aganglionic colon (by enlarging the graph between 0 to 0.3, and reporting the control colon with a break). SD are not present.

We have now modified the Figure.

• Figure legend: please add a sentence describing data, as "data represent the means +/- SD of SK3 expression normalized to GAPDH and relative to control tissues).

We have now added the following sentence in the Figure legend: “Data represent the means ± SD of SK3 expression normalized to GAPDH and relative to control tissues.”

• It might be of interest to perform a real-time PCR to test the level of expression of PDGFRA as a control and a marker for the PDGFRA+ cells, supposed to express SK3 and to have a role in persistence of bowel symptoms

We have now added the following sentences in the Discussion section: “It might be necessary to determine the PDGFRA expression level as a control and a marker for the PDGFRA+ cells that express SK3 for better understanding the persistence bowel symptoms pathogenesis in HSCR patients following pull-through procedure.”

• Please comment on the difference of percentages of patients with reduced SK3 expression found in the present research with previous publications. To note, Coyle and coll. found a SK3 down-regulation in only half of analysed patients. Differences maybe attributed also to differences in the genetic background of analysed populations (Asiatic vs Caucasian), different surgery procedure, others.

We have now added the following sentences in the Discussion section: “However, it should be noted that previous study (Coyle et al., 2015) showed only half HSCR patients had reduced the SK3 expression. These differences might be attributed to the differences in the genetic background of analyzed population between previous report (Coyle et al., 2015) versus our study.

• Discussion lines 171-174. This sentence is too speculative. Please note, Your data do not contribute with any genetic data relevant to the genetic counselling. In my opinion, it is
appropriate to conclude that the results reported may contribute to extending the knowledge on mechanisms causing the persistence of bowel symptoms. Please, modify this sentence.

We have now modified the Discussion section lines 171-174 to the following sentences: “This study may contribute to extending the knowledge on mechanisms causing the persistence of bowel symptoms after an appropriately performed pull-through.”

- Discussion, line 174-176. The explication on the possibility of continuing intestinal symptoms does not properly concern the "genetic counselling", more appropriate a "surgery counselling". please, modify this sentence.

We have now revised the Discussion section lines 174-176 to the following sentences: “The possibility of the continuing intestinal symptoms after an appropriately definitive surgery should be explained during surgery counseling to HSCR parents.”

Minor points:
- Methods, line 105: please use the term "SK3 expression", instead of SK3 expressions, was…. the same in line 131

We have now used the term “SK3 expression was” in lines 105 and 131.

- Results: line 123: because of

We have now used the term “because of” in line 123.

- Discussion: line 169: please eliminate one "that".

We have now eliminated one “that” in line 169.

- Needs some language corrections before being published

The manuscript has been reviewed for grammar and proofread by a native speaker who is fluent in scientific English.
Reviewer #2 (V.G. Shakkottai):

In this article by Gunadi et al. expression of SK3 channels is assessed in ganglionic and aganglionic segments of cases of Hirschprung disease. The study is interesting and reproduces findings from a prior report in a different population.

We appreciate the encouraging comments of this reviewer.

The study could be improved with the following changes.

1. It is important to note that the reduction in SK3 occurs in addition to other neurotransmitter signaling in disease. The authors give the impression that the changes are restricted to SK3.

We have now added the following sentences in the Discussion section: “It is important to note that the down-regulation in SK3 occurs in addition to other neurotransmitter signaling in HSCR.”

2. The control population demographic information should be provided in table 1. This is important to know in order to determine how well the samples were matched.

We have now added the controls demographic information in the Table 1.

3. A major contention of the authors is that SK3 reduction in the ganglionic segment predicts persistence of bowel symptoms. This is not addressed at all as a follow up to the cases. It would be informative to present data on how patients did following surgery and whether the reduction in SK3 in the ganglionic or aganglionic segment in individual cases influences outcome. If long term follow up data are present, these should be included.

We have now added the follow up of HSCR patients in the Results section: “We followed-up all patients for a mean of 10.8 ± 16.0 months following pull-through. Three individuals (patient 4, 6, and 11) developed enterocolitis in 5, 18, and 2 months, respectively, after pull-through, while only one subject (patient 6) suffered post-operative soiling (Table 1). All symptoms resolved with rectal irrigations and administration of oral metronidazole.”

4. It could be helpful to show immunostaining from the resection segments for SK3-this would also give the reader more confidence that the appropriate segments of gut were analyzed by qPCR.
We have now added the following sentences in the Discussion section: “Unfortunately, we do not have any data on immunostaining of the resection segment for SK3 due to limitation of resources in our institution.”

5. Some more information about sample preparation would be helpful. Were samples examined by conventional histology and adjacent gut used for RNA extraction? How much tissue was used for RNA isolation? How did the authors ensure that the sample for RNA isolation was from aganglionic (or ganglionic) colon?

We have now added the following sentences in the Methods section: “Intraoperative pathological evaluation was performed during pull-through procedure to ensure that the sample for RNA extraction is from aganglionic (or ganglionic) colon.”

“Total RNA was isolated from 25-30 mg of colon tissue using the total RNA Mini Kit (Tissue) (Geneaid Biotech Ltd., New Taipei City, Taiwan).”

6. Needs some language corrections before being published

The manuscript has been reviewed for grammar and proofread by a native speaker who is fluent in scientific English.