Author's response to reviews

Title: Beta catenin and cytokine pathway dysregulation in PTEN hamartoma tumor syndrome patients

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Title: Beta catenin and cytokine pathway dysregulation in "PTEN hamartoma tumor syndrome" patients

Dear Editor,

Thank you for your letter dated December 21, 2012 concerning the article: "Beta catenin and cytokine pathway dysregulation in PTEN hamartoma tumor syndrome" by Martina Galatola et al., that we submitted for publication.

We have revised the manuscript taking into consideration all the points raised by the reviewers (see point-by-point Response to Reviewers).

We hope that, having complied with the requests of the Reviewers, our paper is now acceptable for publication.

Looking forward to hearing from you,

Yours sincerely,

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Response to Reviewer 1: Prof. Maurizio Genuardi

MAJOR COMPULSORY REVISIONS

1. While a clinical diagnosis of PHTS is reasonable for case 1, the manifestations reported for cases 2 and 3 are less convincing, especially for patient 3. Hence the causal relationship with a PTEN defect, particularly given that this is an RNA alteration without a demonstrated DNA defect, is only hypothetical. The main clinical manifestation in case 3 is hamartomatous polyposis: were other conditions associated with hamartomatous polyposis excluded? The association with a congenital malformation (horseshoe kidney) is more suggestive of juvenile polyposis, especially in the absence of additional typical PHTS manifestations. Was genetic testing for SMAD4/BMPR1 performed?

We have not performed the genetic testing for SMAD4/BMPR1, taking into consideration the clinical differential diagnosis with Juvenile Polyposis syndrome, made from endoscopist and histologist. This differential diagnosis is mainly based on the histological characteristic of the amartomatous polyps of these patients and on other clinical characteristics, such as glycogenic acanthosis.

We precised this point in the revised version of the paper.

Methods. Page 5, Line 4

“…The histological aspect of the polyps were unambiguous in all cases and it is tipic of PHTS polyps. Differential diagnosis with the juvenile polyposis Syndrome (JPS) was made for PHTS2 and PHTS3 patients because none of the patients showed polyps histologically characteristic of the JPS. In addition, the PHTS2 patient showed extensive glycogenic acanthosis, symptom typical of Cowden syndrome…."

2. The authors should provide more compelling evidence for considering the reduced PTEN expression levels in cases 2 and 3 as indicative of an undetected constitutional defect/mutation. For instance, genomic deletion analysis was performed using a single probe located in intron 9. More thorough analysis, by MLPA or by more extensive Real Time PCR with multiple probes, should be performed to rule out the possibility of large genomic rearrangements that can reduce mRNA stability or involve the region containing exon 5-6 junction, that was specifically tested with the RT-PCR probe. Mutation/methylation analysis of the 5’ non coding region is also warranted to identify alterations causing reduced expression.

We agree with the Reviewer that more extensive Real Time PCR with multiple probes, should be performed and mutational analysis of the 5’ non coding region is also necessary to identify alterations causing reduced expression. Mutational analysis of the 5’ non coding region have been performed and now reported in the revised version of the paper.

Methods. Page 9, Line 5

“…Mutational analysis of PTEN promoter region, from bp -1398 to bp +1, was performed by PCR and sequencing. This region was amplified into two overlapping fragments of 663 and 789 bp in molecular weight, respectively, using the following primer pairs:

PTENp-FP: GTTTCTCGGCTCTCTTTGGT; [NC_000010.10] start: 89623575;
PTENp-RP: ATGGCTGTCATGTCTGGGA; [NC_000010.10] start: 89624237;
PTENp2-FP CTACACTGAGCAGCGTGGTC; [NC_000010.10] start: 89622825;
PTENp2-RP GGCTGCACCGGTAGAAAAGA; [NC_000010.10] start: 89623613.

……
Regarding the Real Time PCR genomic analysis, we have performed the amplification of an additional fragment encompassing the region between exon5 and IVS5, now reported in:

**Methods. Page 9, Line 14**

For PTEN specific quantification, two short fragments, one inside intron 9 and the other at the boundaries of exon 5 and IVS5, were amplified, using the following primer pairs:

3. Mixing RNAs from different controls does not allow accurate evaluation of interindividual variability in expression. The 20 control samples should be analysed individually, to provide a more realistic estimate of standard deviations. Theoretically, it is possible that some normal samples had mRNA levels in the range of PHTS cases, but this could be masked by the use of control mixtures.

We agree with the Reviewer and we have analysed 20 control samples individually (showed in Figure 1b). and we have included in the text in:

**Methods Page 7, Line 24**

"...In order to allow accurate evaluation of interindividual variability in expression, we also separately analyzed 20 samples, collected from healthy subjects...."

**MINOR ESSENTIAL REVISIONS**

1. **Background, page 3, last sentence of 2nd paragraph: delete “therefore”**

*Background. Page 3, Line 19*

We have deleted “therefore” from the text, that now is: ".... It is recommended that individuals with BRRS should ...."

2. **Methods. Page 5, 1st paragraph: delete 1st sentence “When .. familial cases”, as the concept is obvious.**

*Methods. Page 5, Line 9*

We have deleted this sentence from the text, that now is: “.... Clinical phenotype, family history and molecular characterization of each patient are reported in Table 1. Blood samples from healthy subjects were collected from the same hospital as the PHTS patients......”

3. **Methods: specify how many cells from each subjects were used for western blotting.**

*Methods. Page 10, Line 2*

We specify in the text."...Total proteins were extracted from 3 ml of peripheral blood cells (about 5-7 x 10^3/mL cells) using Trizol reagent...."

4. **Results, page 9, paragraph beginning with “As shown ..”. The order of figures 1c and 1d should be inverted (alternatively, the text description can be inverted). In the same paragraph, “... was a carrier of .... whole gene deletion”: neither promoter mutations/methylation nor partial deletions can be excluded.**

Panel a (and b) of the Figure 1 as been deleted, as suggested from the Reviewer in a subsequent point. Figure 1a and 1b now show PTEN mRNA quantification, both with the four mixes of healthy subject that with the twenty healthy controls separately (Figure 1a and 1b, respectively). Figure 1c shows gene copy number quantification.

**Results. Page 12, Line 8**

Taking in to consideration the second part of the the Reviewer’s point, we have included in the text " .....None of these patients was a carrier of a PTEN point mutation of the promoter or coding gene region, nor did they show intragenic or whole gene deletion when analysed by
Real-Time genomic PCR (Figure 1c). However, promoter iper-methylation cannot be excluded, and additional mechanisms could be responsible for the PTEN…….

5. Results, page 10: it is interesting that the reduced APC levels are found in the two cases that do not have a PTEN mutation identified on DNA. This could be due to alternative mechanisms (possibly mutations in other genes), that cause reduction in both APC and PTEN mRNA levels.

We agree with the Reviewer and we have reported in the revised version of the paper in: Discussion. Page 16, Line 14
"….It is interesting that the reduced APC levels are found in the two cases that do not have a PTEN mutation identified on DNA. We can not exclude that this could be due to alternative mechanisms (possibly mutations in other genes), that cause reduction in both APC and PTEN mRNA level….."

6. Results, pages 10-11. The first few sentences of the section entitled “Cytokine dysregulation is observed ….” (where “cytochine” should be changed to “cytokine”), from “Finally ….” to “Moreover, many literature data correlate with ….” contain general concepts that are more appropriate for the introduction or the discussion.

Discussion. Page 14, Line 18
We have changed cytochine with cytokine in all text. In agreement with the Reviewer’s comment we have discussed this concepts in the discussion section:
“It is becoming increasingly evident that inflammation and cancer are intricately related. Many cancers arise from sites …….."

7. Discussion, page 13, 1st paragraph, 2nd sentence: “The authors showed …. PGE2 production”. Please clarify.

Discussion. Page 15, Line 26
We have clarified this point in the text: “….The authors showed that Akt phosphorylation was high in mutated PTEN cells compared to wild-type PTEN cells, and that this phosphorylation status is associated with overexpression of COX-2 mRNA, its protein levels, and PGE2 (prostaglandin E2) production..)

8. Discussion, page 13, 2nd paragraph: the issue of the need for quantitative mRNA screening is certainly important. However, as previously specified, the two patients on which only mRNA level reduction was observed are also those that have a less significant phenotype.

This point has been discussed before, in the answer to the first comment of the Reviewer.

9. Figure 1a should be deleted, as it does not show relevant data.

We have deleted panel a (and b) of the Figure 1, as indicated at point 7.

10. Legend to figure 1c and d (please note the inverted order of 1c and 1d mentioned above): “Control 1 and 2 are two healthy subjects supposedly not deleted for the target genes” replace “supposedly” with “apparently”.

We have replaced “supposedly” with “apparently” in all text.
Response to Reviewer 1: Prof. Anxo Vidal

MAJOR COMPULSORY REVISIONS

1. Methods section should be clarified. The present form that the methods are presented for mRNA and gene copy quantitation, as well as sequence analysis from both cDNA and genomic is confusing. Primers used and the precise regions analyzed by each approach should be clearly stated for best understanding. Also the method for protein extraction is missing and just “following manufacturer’s instructions” is stated, without any other reference. Since functionality of beta-catenin depends on subcellular localization, this may not be a minor issue.

We agree with the Reviewer and we have inserted in the revised version the sequences of the Primers used and the regions analysed. Moreover, we have organized the methods section in the following paragraphs:

**Methods Page 6, Line 4-5**

**Molecular analysis of the PTEN messenger.**

RT-PCR of PTEN full length coding region in PHTS patients.

**Methods Page 7, Line 4**

Sequence analysis of PTEN messenger

**Methods Page 7, Line 17**

Real-time RT-PCR quantification analysis

**Methods Page 8, Line 22-23**

**Molecular analysis of PTEN gene and PTENP1 pseudogene.**

Genomic PCR and sequencing.

**Methods Page 9, Line 12**

Gene copy number quantification of PTEN gene and PTENP1 pseudogene.

**Methods Page 10, Line 1-2**

**Molecular changes of PI3K/Akt and WNT pathways associated to PHTS syndrome**

Western blot assay of b-catenin protein.

**Methods Page 10, Line 17**

Real-time PCR quantification analysis of COX2, CCND1, cMYC, and APC messengers

**Methods Page 10, Line 25**

**Cytokine disregulation on peripheral blood cells of PHTS patients**

Real-time PCR quantification analysis of IL10 and TNFα mRNA.

**Methods Page 11, Line 2**

Western blot assay of TNFRI and TNFRII proteins.

**Methods Page 11, Line 8**

RT-PCR and sequencing of TNFRIαβ.

**Methods Page 10, Line 2**

We clarify in the text the method for protein extraction: “….Total proteins were extracted from 3 ml of peripheral blood cells (about 5-7 x 103/mL cells) using Trizol reagent..”

2. The authors show a missense mutation in one of the patients and lower levels of mRNA in the other two. The molecular explanation for this way of inactivation is unknown but at least it should be discussed. It is clear that sequencing of cDNA can rule out mutations in the coding region as well as in the UTRs, but it is unclear which portion of the promoter (page 9, second paragraph of results) was sequenced. From Methods it looks like “a short fragment inside intron 9 was amplified”, is it enough evidence to disregard the possibility of intragenic deletions? On light of the experimental evidences provided, possible mechanisms for PTEN mRNA should be considered.
We agree with the Reviewer that more extensive Real Time PCR analysis is necessary to disregard the possibility of intragenic deletions. Mutational analysis of the 5' non coding region is now properly expressed in the methods section.

**Methods. Page 9, Line 5**

We have included in the text: “…Mutational analysis of PTEN promoter region, from bp -1398 to bp +1, was performed by PCR and sequencing. This region was amplified into two overlapping fragments of 663 and 789 bp in molecular weight, respectively, using the following primer pairs:

- **PTENp-FP**: GTTTCTCGCCTCCTCTCTCGT; \[NC\_000010.10\] start: 89623575;
- **PTENp-RP**: ATGGCTGTCATGTCTGGGA; \[NC\_000010.10\] start: 89624237;
- **PTENp2-FP**: CTACACTGAGCAGCGTGGTC; \[NC\_000010.10\] start: 89622825;
- **PTENp2-RP**: GGCTGCACGGTTAGAAAAGA; \[NC\_000010.10\] start: 89623613.

About the Real Time PCR genomic analysis, we have performed the amplification of an additional fragment encompassing the region between exon5 and IVS5.

**Methods. Page 9, Line 14**

…For PTEN specific quantification, two short fragments, one inside intron 9 and the other at the boundaries of exon 5 and IVS5, were amplified, using the following primer pairs:….

Moreover, we have discussed in the text the possible molecular mechanisms for the mRNA inactivation.

**Results. Page 12, Line 11**

”….., and additional mechanisms could be responsible for the PTEN down-expression in these two patients. For example, point mutations in gene regions not investigated, such as intronic regions and regions at the 5’ and 3’ end of the gene (not included in our analysis), or mutations in other genes that could regulate PTEN expression, could be present in these patients…….”

3. The authors favor a model where deregulation of the PI3K-Akt signaling is responsible for alterations found in beta-catenin and cytokine pathways. However, PTEN can act through other pathways and no evidence is provided for PI3K-Akt activation. Mechanistic proof would require pharmacologic or genetic manipulation of patients’ PBCs, which could be challenging, but the authors could show, in protein extracts from PBCs, the phosphorylation status of Akt or some of the downstream targets of the pathway. Also, since the proposed functional link between PTEN-PI3K-Akt and beta-catenin is its phosphorylation, phospho-beta-catenin analysis should be performed by western blot. Phospho-specific antibodies are commercially available.

As required from Reviewer, we have performed the western blot analysis using phospho-β-catenin (Ser552) (showed in Figure 2a).

**Results. Page 13, Line 5.**

We have inserted in the Results section: “…As shown in Figure 2a, β-catenin is detectable by using N-terminal antibody and phospho-β-catenin (Ser552) only in PHTS patients; total β-catenin (detected by N-terminal antibody) is absent in all healthy controls analysed, while phospho-β-catenin (Ser552) is detected mainly in PHTS patients and gives a very faint signal in controls. The phospho-β-catenin (Ser552) accumulation favors a model whereby.”

4. The authors say that in TNFRI western the main signal is a 25kDa band that they claim to be a beta isoform of TNFR1A. However, in figure 3b only shows this band and not any others. Does it mean that other forms of TNFR1A are not detected at all? If other bands are detected they should be shown. Also, besides the molecular weight which is the evidence to support identity of this detected form as isoform beta? It seems that showing the data about mRNA expression and sequencing of these isoforms may help.
We agree with the Reviewer; in TNFRI western other forms of TNFR1A are really detected and we show now this image in Figure 3b. Furthermore, we show RT-PCR and sequencing TNFR1Aβ in Figure 3c and 3d, respectively.

MINOR ESSENTIAL REVISION

1. There are some typos that should be corrected, such as “Figure legends” or “cycline D1”. Also I believe the term “Cytochine” used in some section titles is not correct and should be substituted by “cytokine”, the one used in the main title of the article.

We have corrected all typos and submitted the paper to an English reviewer for the language supervision.

2. Figure 1 is mislabeled: panel c) shows genomic and panel d) mRNA quantification.

Panel a (and b) of the Figure 1 as been deleted, as suggested from the other Reviewer. Figure 1a and 1b now show PTEN mRNA quantification, both with the four mixes of healthy subject that with the twenty healthy controls separately (Figure 1a and 1b, respectively). Figure 1c shows gene copy number quantification.

3. The authors show increased levels of messenger for c-myc or cyclin D1 in non-neoplastic blood cells, when in other instances c-myc overexpression is sufficient to drive oncogenesis. The authors could discuss the functional consequences of such alteration regarding tumor predisposition in PHTS patients.


We take the Reviewer’s point and we have discussed it in the text: “….Increased levels of messengers cMYC and CCND1 are often observed in different tumour types, such as breast cancer, endometrial cancer, thyroid cancer and ovarian cancer [38-40]. Interestingly, these types of tumours correspond to the neoplasias most frequently associated to PHTS syndrome…”

Looking forward to hearing from you
Yours sincerely
Marina De Rosa