Author's response to reviews

Title: Can we accurately report PTEN status in advanced colorectal cancer?

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Author's response to reviews: see over
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Dr Milo Frattini
BioMed Central Editorial
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Dear Dr Frattini,

re: MS: 1693199915105585 – “Can we accurately report PTEN status in advanced colorectal cancer?”

On behalf of my co-authors I thank you for the opportunity to submit a revised version of our manuscript. We would like to thank the reviewers for taking the time to review our work so thoroughly. We were pleased both reviewers felt the paper was of interest and an important contribution to the field. We hope the revised manuscript provides an important review of the current limitations of PTEN as a potential biomarker in colorectal cancer and highlights the crucial methodological limitations to its assessment.

We have made extensive revisions to our paper by addressing each of specific reviewer revisions in detail as noted below. We have not undertaken further laboratory studies due to the time constraints on resubmission and importantly we do not feel this would change the overall insight the current manuscript provides. If the editor feels this is essential to proceed with publication we would be happy to reconsider this.

Reviewer 2 had no recommendations and the review was positive.

Reply to Reviewer 1 comments:

Major revisions:

1. In the materials and methods section a brief description of the analysed cohort should be introduced.

A formal description of analyzed cohort has now been included in the methods section.

2. In the materials and methods section (“PTEN copy number variation paragraph”) the authors should explain better some points of the methods and evaluation criteria used. More specifically:
   a) Report the sequence of the primers used for PTEN copy number variation analysis and the PTEN gene region covered by the assay.

      The sequences of the primers were not available as this is proprietary information of Life Technologies. We have included the gene region and location of the primers in the Methods section.

   b) Explain how the PTEN copy number of the cell lines used as controls is known. The authors have to test the PTEN copy number in these cell lines also with other methods (e.g. FISH) to validate their methodology. If this has already been done, it has to be specified in this section.
We have expanded on the cell line controls and referenced the paper confirming HT29 (the primary control) has 3 copies of chromosome 10.

The method used to determine the 3 copy control was spectral karyotyping as per the added reference (Abdel-Rahman et al., 2000). This has been added to methods and the altered section is highlighted, page 8.

c) Explain better and with more details the scoring system used to assess PTEN IHC staining specifying for each score the cut off values basing on staining intensity and on percentage of tumor cells. The authors must specify also if they evaluate the staining of PTEN as localized in the cytoplasm, in the nucleus or both.

Further detail has been added to explain the IHC scoring system used. The % of cells stained was not used as part the chosen scoring criteria. One of our aims is to highlight the lack of standardised scoring systems in the literature.

3. The authors consider as PTEN loss by IHC those cases scored as zero. Also the cases with a reduction in PTEN expression should be considered and evaluated for the concordance between IHC and CNV assay. In fact in the evaluation of copy number variation the Authors consider as PTEN loss those cases with # 1.5 copies of PTEN gene, thus meaning that they consider as loss also the cases maintaining intact one allele, which, if it is not altered through other genetic alterations, could be transcripted and translated in a well functional protein. In this case, a reduction but not a complete loss of PTEN protein expression could be seen in IHC. A reduction of PTEN expression could be in fact the effect of the PTEN haploinsufficiency whose role in providing tumor growth advantage is a matter of debate. In your cohort there are 14 cases with a loss of PTEN detected by CNV analysis but with no loss in IHC. Maybe in these cases there is a reduction in PTEN protein expression and not a complete loss.

On the issue of whether the definition of PTEN loss on IHC should include 'reduced staining' (pts with 0 and 1+ staining rather than only 0 staining). There is no consensus in the literature on this issue. Loupakis 2009 and Park 2011 used a definition of PTEN loss which included patients with 'reduced' staining but included high intensity staining (3+) in <25% of cells and moderate staining (2+) in 25-50% of cells as 'PTEN loss'. This is in contrast to Laurent-Puig, JCO, 2009 where no staining in any cells at any intensity was required to define 'PTEN loss'; consistent with our definition. This explanation has been added to the second last paragraph of the introduction.

We have added further clarification to paragraph 1 of discussion to highlight the correlation between IHC loss and allelic loss and raise the uncertain functional implications of monoallelic loss.

4. It is not clear if the authors, for the analysis of PTEN CNV, normalise the results only with the reference gene (RNase P) or also with a calibrator sample (a sample containing 2 copies of the target sequence). For the evaluation of gene copy number
variation through a TaqMan assay is fundamental to normalize the Ct value of the target sample against both a reference gene and a calibrator that could be, in an ideal situation, the corresponding normal tissue of the tumor sample in analysis.

Yes we have normalized the results with a calibrator sample. The Rotorgene software normalizes against both the reference gene and the diploid calibrator using the $2^{-\Delta\Delta\text{Ct}}$ method. The calibrator used for these calculations need not be the corresponding normal tissue but any cell line or tissue with 2 copies of PTEN, in this study we used the cell line LIM1899

5. The cell lines used as controls for CNV assay should be analysed also for PTEN by IHC in order to check for inter-observer variability in this simpler setting and to check for the concordance between the two methodologies.

We note the suggestion of using cell line controls for CNV assay also for PTEN by IHC to check for inter-observer variability and concordance between the two methodologies. While this would be an interesting additional study we do not believe that this kind of comparison will add to the present study of IHC on heterogeneous tumor tissue.

6. It is not clear what is the meaning of the Majority Score used to compare IHC and CNV results. Only cases resulted concordant for PTEN immunohistochemical evaluation between the two pathologists should be considered.

As per the reviewers suggestion, we have changed the analysis of IHC vs CNV to include only specimens which were concordant between pathologists on IHC assessment. Table 2 has been amended accordingly.

This change has meant the overall majority IHC score is no longer required (and hence removed from table 1). The individual pathologists 'majority score' of IHC readings (majority of three sections of tumour) has been clarified in the methods IHC section.

7. The five specimens in which pathologist JC didn’t find tumor cells have not been considered for inter-observer variability and also for CNV analysis.

The IHC concordance rate includes the cases where no tumor was identified. This has been clarified in the results section (IHC). We have censored these for the purpose of IHC vs CNV analysis as only the concordant IHC results have been used for this analysis (see point 6 above).

8. Cases in which the PTEN IHC evaluation was discordant between the two pathologists should be revised and maybe re-evaluated on the entire tumor section.

The reviewer suggests cases of IHC discordance between pathologists be revised. We feel resolving the discordance amongst these 19 specimens will not add to the overall message of our manuscript which aims to highlight this discordance. We have raised this in the discussion paragraph 2. Our manuscript does not aim to define the best IHC scoring system, only to highlight the limitations of all current PTEN assessment methods.

9. In the discussion section the Authors must discuss better and critically the results obtained, by giving possible reasons for explaining the differences found between
the two methodologies, focusing mainly in providing possible explanation about why 14 cases showed loss of PTEN expression by CNV analysis and not in IHC and why 6 cases showed loss of PTEN expression in IHC and not in CNV analysis.

Thank you for requesting further explanation of the IHC/CNV discordant results. The discussion (paragraphs 1-3) has been extensively revised. Indeed we feel the alternate genetic mechanisms and thus relevant analytical techniques is a key message of the manuscript.

10. In the discussion section, the references and examples taken from the literature must serve to discuss and support the results obtained and so they must be integrated better in the text by discussing the data from other papers reporting the inter-observer variability in IHC assessment and the comparison of IHC with other methodologies. In the paper of Sangale and colleagues (Sangale et al. Appl Immunohistochem Mol Morphol 2011;19:173-183), the authors developed an optimized IHC assay and they found 100% concordance between 3 independent pathologists. Moreover, in the discussion section, the part regarding the prognostic and predictive value of PTEN should be better integrated in the text.

Paragraphs 4-5 of the discussion have been revised to include discussion of available data on inter-observer variability of IHC. We have included the important reference of Sangale et al on an optimized PTEN IHC assay. The section discussing the conflicting evidence of predictive and prognostic role of PTEN has been simplified as a thorough discussion of these papers is beyond the scope of this paper.

Minor revisions:

1. Introduction: in addition to retrospective analyses, very recently, an exploratory biomarker analysis of the 20020408 clinical study demonstrated that mutations in exon 2 and 3 of N-Ras gene were linked to resistance to panitumumab treatment in metastatic colorectal cancer patients (Peeters M, et al. Clin Cancer Res 2013;19:1902-1912)

This has been changed to RAS mutation and references (Peeters and Douillard) added.

2. Introduction (page 4, 1° paragraph): when the authors are explaining the several mechanisms leading the PTEN loss of function, there is an incongruity between the occurrence of PTEN mutations (2-12%) and the percentage of PTEN protein loss in IHC the mutations should account for (19-54%).

PTEN mutation here refers to "small-scale mutations" ie point mutations, deletions or insertions. This has been added for clarity.

3. Introduction (page 4, 1° paragraph): in the paper of Loupakis (ref 15, mentioned in the text), the authors analysed both primary tumor5 and metastasis for PTEN protein status. It is not true, as written in the text, that Loupakis and colleagues have analysed exclusively primary CRC.
The reference of Loupakis and others demonstrate the discordance between primary and metastatic specimens. This has been clarified in the text.

We hope that we have responded appropriately to the reviewer’s queries. We would, as noted above, be happy to review again if felt necessary. We will now await your further review and consideration of the revised manuscript.

Yours sincerely

Timothy Price