Reviewer's report

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

Version: 2

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Reviewer: Francesca Molinari

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In the manuscript "Can we accurately report PTEN status in advanced colorectal cancer?" by C. Hocking, the Authors evaluated PTEN copy number variation (by Taqman assay) and protein expression (by IHC) in 56 colorectal cancer specimens in order to analyse the inter-observer variability of IHC evaluations and the concordance of PTEN status between the two methods used to analyse PTEN. The authors reported a discordance both in the PTEN immunohistochemical evaluation by two different pathologists and in the results obtained by analysing PTEN through IHC and copy number variation assay.

The issue of this paper is important and interesting as there are not, at the moment, standardized methods for PTEN evaluation and IHC analysis suffers from inter-laboratory and inter-observer variability.

Major Compulsory Revisions

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

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:

   • Report the sequence of the primers used for PTEN copy number variation analysis and the PTEN gene region covered by the assay.
   
   • 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.
   
   • 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.

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.

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.

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.

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.

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

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.

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.

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.

Minor Essential 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)

2. Introduction (page 4, 1° paragraph): when the authors are explaining the several mechanisms leading to 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%).

3. Introduction (page 4, 1° paragraph): in the paper of Loupakis (ref 15, mentioned in the text), the authors analysed both primary tumor and metastasis for PTEN protein status. It is not true, as written in the text, that Loupakis and colleagues have analysed exclusively primary CRC.

For all these reasons the paper cannot be accepted in the present form and must be rejected.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:

I declare that I have no competing interests