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
Dear Editor,

Thank you for again inviting us to submit further revisions to this manuscript. For Reviewer 2, we note they are happy with all revisions made thus far and there were no new suggestions. Reviewer 1 has raised two important points of clarification and we hope we have clarified their questions. We have addressed each suggested revision in detail below and highlighted these changes in yellow in the text.

Major Compulsory Revisions

1. An explanation on how the PTEN copy number of the HT-29 was known have been added in the text. The same has to be done for the LIM2405 and LIM1899 cell lines as these cell lines, together with the HT-29 cell lines, have been used to validate the PCR assay and so you have to be sure that the LIM2405 and LIM1899 contain 1 and 2 copies of the PTEN gene respectively. Moreover, the LIM1899 cell line has been used as calibrator and so the PTEN copy number of this cell line, and also of the LIM2405, must have been established also by other methods (in the literature or by your previous studies).

2. The authors must include in the material and methods section that they use as calibrator for CNV analysis the cell line LIM1899, on pending demonstration of the presence of 2 copies in the cells with another methodology (See comment above). This information is important to understand how the calculations of 2-##Ct has been done.

I will address revisions 1 and 2 together as they both relate to establishing the number of PTEN genes in cell lines LIM2405 and LIM1899.

We used the known HT-29 cell line as a control to calculate the CNV for LIM2405 and LIM1899 using PCR analysis. This is outlined in the methods section as follows, “We tested DNA from colon cancer cell lines to determine the reproducibility of the assay and to select cell lines to use as copy number controls. HT29 (ATCC) is known to have 3 copies of chromosome 10 as determined by
spectral karyotyping and comparative genomic hybridization and was used as the primary control sample for 3 PTEN copies. Cell lines LIM2405, LIM1899 (both a kind gift from The Ludwig Institute, Melbourne) and HT29 were tested in quadruplicate and repeated in 3 separate PCR assays. The assay was both precise and reproducible - the means for LIM2405, LIM1899 and HT29 were 1.08 SEM 0.04, 2.07 SEM 0.03 and 2.96 SEM 0.07 respectively, and the coefficient of variation (CV) from run to run was 2.4%, and intra-assay CV was between 0.12% and 0.99%. These cell lines were therefore used as 1-, 2- and 3-copy controls respectively.

Our group has previously published this analysis and the reference to this publication has been added to the methods section.

- see Pg 8, highlighted in yellow.

3. Regarding the five specimens in which only the pathologist JC didn’t find tumor cells, I return on the point that these cases have to be excluded from the analysis or re-evaluated for the presence/absence of tumor cells. You have replied me that the IHC concordance rate includes the cases where no tumor was identified but in the material and methods section is written that the cases where selected on the basis of the presence of tumor cells. Moreover it’s not possible that two pathologists, on the same section, found different things as regard the presence or absence of tumor cells. If the pathologists have evaluated different sections, it could be possible that one pathologist found tumor cells and the other one not on a different slide, but in this case, you cannot use your results for inter-observer variability assessment, because, for inter-observer variability studies, you have to evaluate the same slides. Please, clarify this point.

Thank you for raising this important point. We can confirm the two pathologists reviewed the same slides. As such we agree the specimens with a question of tumour present or not should be excluded from the analysis (see first paragraph of results section) and have done this as suggested. We have changed the tables and re-calculated the numbers throughout the paper to reflect this.

-see table 1 and numbers throughout text, highlighted in yellow

Minor Essential Revisions

1. Introduction, 1° paragraph: the sentence “In addition to KRAS, mutation of genes involved in downstream EGFR signalling pathways Ras/Raf/MAPK and PIK3CA/AKT also confer resistance to anti-EGFR MoAbs” has to be changed in “has been proposed to confer” as the role of BRAF, PIK3CA and PTEN has not been validated yet.
2. Introduction, 1° paragraph: in the sentence “Specifically, mutations in BRAF and NRAS genes...”, remove NRAS (as you have added in the text above together with KRAS) and you can replace with PIK3CA gene for which has been proposed also a role as predictive marker.

This change has been made
- pg 3 highlighted in yellow

3. Introduction, 4° paragraph: in the sentence “other groups have assessed PTEN loss using FISH etc...) do you mean other groups studying the PTEN predictive value? If yes please add in the text. Moreover by reading this sentence it seems that with FISH analysis you can detect both PTEN loss, mutation and methylation, so this sentence has to be rewritten better.

This has been clarified.
- pg 5 highlighted in yellow

4. Introduction, 5° paragraph: I think there is a typing error: “taqMan and ref”

This has been deleted

5. Results, last paragraph: in the description of how many cases have PTEN allelic loss in Taqman and in IHC, please add also that 15 cases which do not have PTEN loss both in IHC and Taqman because this information cannot be obtained by the present description.

A sentence detailing these 15 cases has been added.
- pg 9 highlighted in yellow

6. Discussion: in the first sentence you would add the inter-observer variability as the purpose of the study is in first to evaluate the interobserver variability and next the comparison of PTEN status with two different methodologies.

This has been added to the text.
7. Discussion, 2° paragraph: the percentage of 32% is wrong: 7 (IHC positive expression)/17 (allelic loss) = 41%

This correction has been made, thank you for pointing this out.

8. Discussion 5° paragraph: thank you for adding the reference of Sangale and colleagues. As they found 100% concordance between 3 pathologists you have to discuss your discordant results giving possible explanation of this discordance.

For all these reasons the paper can be accepted pending revision

We have added brief discussion on the causes of discordance in our analysis.

We look forward to your response to our clarifications and advice from here.

Yours sincerely

A/Prof Timothy Price