Author's response to reviews

Title: Clinicopathological features of KRAS, NRAS, BRAF and PIK3CA mutations in Japanese patients with metastatic colorectal cancer

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Author's response to reviews: see over
Dear Editor-in-Chief,
Dr. Dafne Solera,

Please find enclosed the revised manuscript entitled “Clinicopathological features of KRAS, NRAS, BRAF and PIK3CA mutations in Japanese patients with metastatic colorectal cancer” for consideration as a Research Article in BMC cancer. We have corrected our original manuscript according to the reviewer’s comments and have responded to each comment as follows:

Reviewer: Anders Edsjö

The research question should be clearly stated in the abstract.
→Reply: As the reviewer suggested, we additionally described the research question in the abstract on line 29-32 in the revised manuscript.

The authors should describe the tumor material in higher detail. It should be clear whether primary tumors and/or lgl or distant metastases have been analyzed. To be able to assess whether the assay had a chance of detecting mutations, a knowledge of the percentage of neoplastic cells in the tumor samples is needed. This could either e.g. be clarified by the authors stating the lower limit accepted by the study.
→Reply: As the reviewer suggested, we additionally described whether primary tumors or distant metastases have been analyzed on line 113-114 in the revised manuscript. We have no information of exact percentage of neoplastic cells in the tumor samples, but our institutional pathologists confirmed the existence of adequate cancer cells and selected most appropriate FFPE section to test genome analysis. Considering the sensitivity of our method, the lower limit of the percentage of mutant allele in the tumor samples accepted by the study was 5%. We added this sentence in the method part on line 121-122 in the revised manuscript.

The authors should also state the level of detection in terms of allele frequencies needed for the refractory mutation system-Scorpion assay used.
→Reply: As the reviewer suggested, we additionally described the sensitivity of the refractory mutation system–Scorpion assay on line 130 in the revised manuscript.

The yellow parts of figs. 1-2 are difficult to read if printed on a regular printer. It might be a good idea to test other combinations of colors or symbols.
As the reviewer suggested, we changed the combinations of colors of figure 1 and 2 in the revised files.

A file with the genotype of all samples using HGVS nomenclature as supplementary material would be of interest to (some of) the readers.

As the reviewer suggested, we attached the additional file 1 including the genotype of all samples using HGVS nomenclature and added the sentence “The genotypes of all samples using HGVS nomenclature are shown in Additional file 1.” on line 176-177.

The authors are advised to rephrase the passage on assay characteristics on line 247. Although used by others, the terms sensitivity is potential misleading as it has nothing to do with the generally accepted terms? sensitivity? and? specificity? used to characterize the ability of medical assays to detect the condition they are design to detect. A more appropriate term might be ?level of detection? or ?allele frequency needed to detect??

We used terms “sensitivity” according to previous reports below1,2, although the reviewer comment was also understandable. Hopefully these terms are better to be standardized in the near future.


**Reviewer : Javier Hernandez-Losa**

The authors employed a multiplex assay kit that had been previously published with perfect correlation with a comparative arm (sanger sequencing). Comparing the results from both manuscripts with different number of patients, in the present study the patients got better results in terms of FPS and OS. These differences could be explained by the different anti-EGFR therapy?(Cetuximab or Panitumumab).

We considered that the slight difference in PFS and OS was not due to the different anti-EGFR therapy but to the relatively the small sample size. This issue should be analyzed in a larger cohort. Now we are conducting a nationwide genomic screening project for advanced GI patients using this panel.

The authors show in the discussion part, that the frequency of the mutations (KRAS exon 3 and 4, and NRAS 2,3 and 4) is lower that other Western reports. The authors
reflect a total of 12.1% meanwhile in the abstract refer that 34.1% of KRAS exon 2, 3.8% KRAS exon 3 and 4 and also 4.2% in NRAS mutant, resulting a 8% of mutant frequency without KRAS exon 2 in their population. Please comment this discrepancy.

*→Reply:* Among 264 patients (all patients), mutations in KRAS exon 2 and other RAS mutations were detected in 90 (34.1%), 21 (8.0%), respectively. Thus, a total of 12.1% of patients without KRAS exon 2 mutations had other RAS mutations (21/174=12.1). We added this sentence in the abstract on line 40 in the revised manuscript.

On the above comment, the authors propose several explanation for this lower mutation frequency including technical issues. One of them is the sensitivity of the assay that refer 5-10% whereas other recent publication using HRM provide 12% of mutant in japanese population "High-throughput screening of extended RAS mutations based on high-resolution melting analysis for prediction of anti-EGFR treatment efficacy in colorectal carcinoma. Clin Biochem. 2014 Sep 28". Please comment this discrepancy in the discussion part.

*→Reply:* As the reviewer mentioned, one report in Japan showed that other RAS mutations were detected in 7 (12.7%) of 55 samples without KRAS exon 2 mutations, similar to our result. In this article, they describe the sensitivity of HRM as follows: Performing the serial dilution study with control plasmid, the detection sensitivity of mutation was as follows: KRAS (exon 3, 6%: exon 4, 13%); NRAS (exon 2, 3%: exon 3, 6%: exon 4, 4%, 6%) (data not shown). Therefore sensitivity of their HRM method is almost compatible to that of our method.

We added the sentence on line 249-251 in discussion as follows with citation of Clin Biochem. 2014 Sep 28 : Previous another report in Japan showed that other RAS mutations were detected in 7 (12.7%) of 55 samples without KRAS exon 2 mutations with 3%-13% sensitivity [26], which was similar to our result.

Several possible explanations for the relatively lower frequency of other RAS mutations in these studies compared to Western reports were discussed in further section (on line 251-268).

**Reviewer : Maria Carmela Piccirillo**

All the questions were well defined and methods of the analyses were well described. However, they never declared that the study was retrospective, while this must be clearly stated when describing the study design in both the abstract and full text.

*→Reply:* As the reviewer suggested, we recorded variables’ values backward in time. We
changed the phrase of study design in both the abstract and full text from an observational study to a retrospective observational study.

The authors clearly stated some limitations of the study, such as the small sample size, the single-centre population, the risk of a selection bias (no all the consecutive patients enrolled, but only those evaluated for RAS status in the examined time). They also should clearly state that their analyses were explorative and hypothesis generating (no predefined hypothesis was reported). This could render reasonable the fact that they did not adjust the alpha error value for multiple comparisons.

—**Reply**: As the reviewer suggested, we additionally described that our analyses were explorative and hypothesis generating on line 314-315 in the revised manuscript.

The statistical method applied to test the significance of the differences between the examined groups should be added to the footnotes of the tables n. 1, 2, and 4.

—**Reply**: As the reviewer suggested, we added the statistical method applied to test the significance of the differences between the examined groups to the revised tables n. 1, 3, and 4.

4. Some details are lacking in the statistical methods section:

? The authors should explain the stastistical method applied to compare median age between examined groups

? The method to analyze objective response rates according to mutational status was not described and not inferable (logistic regression?)

—**Reply**: As the reviewer suggested, we added the statistical method applied to compare median age between examined groups (Mann–Whitney’s U test or Kruskal–Wallis test) and objective response rates (Fisher’s exact test) on line 163-165 and tables n. 1, 3, and 4.

The title and abstract convey what has been found, but I suggest to explain in the title the study design (A retrospective observational study of clinicopathological features...)

—**Reply**: As the reviewer suggested, we added the study design in the abstract (on line 33), the methods part (on line 98) and the title(on line 1-4).

Thank you for your consideration. We look forward to hearing from you soon.
Sincerely,
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