Author's response to reviews

Title: KRAS mutations: variable incidences in a Brazilian cohort of 8,234 metastatic colorectal cancer patients

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Author's response to reviews: see over
Dear Editor,

Please find enclosed our revised manuscript titled “KRAS mutations: variable incidences in a Brazilian cohort of 8,234 metastatic colorectal cancer patients” for publication in the *BMC Gastroenterology* journal. We would like to thank referees for the careful and constructive reviews.

We have made some changes based on the referees’ comments (in *italic*), which are detailed below:

**Referee 1 (Reviewer: Timothy Price)**

*Comment 1:* The authors present data from a large Brazilian KRAS registry of sorts which has its origins as a Merck funded KRAS program. The program has no relationship to prescribing anti-EGFR agents, as is the case for similar programs in other countries. It does however provide the opportunity to analyse a large data set as the authors have done. As such this data is of interest particularly to local specialists, and does confirm historic data for exon 2 KRAS mutations and that the ratios are similar in Brazil. Findings related to sex are also of interest.

*Answer:* We appreciate and thank you very much for considering our work of interest.

*Comment 2:* Although I understand that this database pre dates the recent all RAS WT data, it does lead to this now being somewhat out of date. The authors should at least note this significant shift in the RAS story with reference to recent publications eg. PRIME and PEAK studies, together with early data from the FIRE study. Whether it is feasible to run the exon 3,4 and NRAS is also a question to be raised but given the funding implications it would be accepted that this may not be possible.

*Answer:* At the time we started the analysis of our cohort, codons 12 and 13 were the main focus of research based on data related to the response to EGFR treatment in colorectal cancer (Allegra, CJ *et al.*, 2009, our reference 15). Thus, the program sponsored the analysis for codons 12 and 13. PRIME, PEAK and the FIRE studies were performed after our analysis was already in progress. Nowadays we acknowledge the importance of exons 3, 4 and of the NRAS isoform, however we already performed all the analyses for all the 8,234 patients. Nevertheless, in the future we could perform further analyses of the exons 3, 4 and NRAS on a different study.

*Comment 3:* I accept that the female to male ratio is statistically different but I must say the numbers do not differ greatly. The hypothesis is however of interest and the discussion of this is novel. Clarification of the hypothesis may be useful however. Are the authors suggesting that estrogen has a different impact on receptors in the bowel, that is that ER alpha is dominant, ER beta as protective less so. Or is there a difference that then leads to
Answer: Although the difference between male and female patients is not dramatic, there is still a statistical difference where we observe a higher percentage in women compared to men, thus we believe it is valid to point it out in our article. Also this is the largest cohort that shows this difference. Regarding hormonal influence, your questions are extremely interesting and we would like to have all the answers, however at the moment we can only speculate about it. We believe menopause and andropause could have an influence but we cannot affirm it since we did not perform specific experiments that could support this hypothesis. To address the hormonal influence, further studies would be necessary.

Comment 4: The references for relevance of initial findings of KRAS exon 2 MT as a predictor of resistance should include Karapetis et al NEJM as this is a definitive paper.

Answer: The reference was added to the manuscript as recommended, being number 3. Thank you for the suggestion.

Comment 5: G13D mutation rate as expected but not discussed.

Answer: We acknowledge we should have added more information regarding codon 13 to the discussion, thus we have added the following sentences and references to the discussion (page 11): “In relation to codon 13, some studies have shown that mutations in this codon could be less aggressive than in codon 12 and that patients with KRAS Gly13Asp mutant tumours could benefit from anti-EGFR therapies [28, 29]. In addition, recent in vitro studies have confirmed that Gly13Asp mutations are associated with sensitivity to anti-EGFR antibody treatments [30, 31].”

In our study we have shown that mutations in codon 13 are present, however we were unable to provide prognosis comparisons since we could not retrieve the follow up from many patients in our cohort.

Comment 6: Does the Southeast data from the “most developed” part of Brazil reflect clinicians submission of patients material, and therefore varies based on difference in numbers ie if the funding for agents also differs will there be a difference in relevance of the results?

Answer: Although people from the Southeastern part of Brazil have more access to oncology centers, our number of patients is high enough to suggest consistent results regarding percentage of mutations per region. Also, the different proportions of patients per region were taking in consideration to statistically analyze the data.

Comment 7: Re prognosis, which is commented on in the discussion (ie unable to assess), it is unfortunate that survival could not be assessed and I presume there is not a central cancer registry that could have been used for at least this outcome?

Answer: Since the study was sponsored by Merck including several centers across the country, unfortunately we have no access to follow up data.
Comment 8: The authors comment on different rates of MT based on patient background, re this point, it may be useful to the reader to understand the general background of Brazil population. This is relevant when the authors reflect on data from Asia in the discussion.

Answer: References 16 and 17 are related to the general background of Brazil and we have added a more detailed description of the Brazilian ancestry in the Results section (part: Analysis of KRAS according to regions, page 8).

Referee 2 (Reviewer: Iris Nagtegaal)

Thank you very much for the useful comments and for the acceptance to review our work.

Comment 1: in the introduction there is a large paragraph on ER, and relation to CRC. This is not relevant in the introduction, so a summarised part might be replaced to the discussion. However, since the manuscript does not investigate ER status I think this is speculative.

Answer: We have transferred the paragraph on ER from the introduction to the discussion as suggested. So, although we were just speculating (not affirming) the reasons by which hormones could be associated with the differences in percentage of KRAS mutations in relation to gender, we believe an explanation on the association between hormones and colorectal cancer is valid for the reader.

Comment 2: - figure 1 is incomplete
Answer: Thank you very much for the observation. The figure is now complete.

Comment 3: - although there is a significant difference between women and men in the frequency of KRAS mutation, this difference is rather small.
Answer: We apologize if in the text sounds like the differences observed were dramatic, however the percentages are still different and we believe it is statically valid to mention this in the article based on our large cohort.

Comment 4: - in the part about the age distribution the authors discuss all results as if these are significant, however, this is not the case and I think that it is not realistic to state that an opposite results is obtained in these cases.
Answer: We observed differences and we explained that the p value is p>0.05 for the ages 60-70 and above 70 and that further studies should be performed to confirm our observation. So, we are sorry if the text might sound too affirmative, however we mention in the text that further studies are needed.
Comment 5: At least part of the data could be gathered in figures rather than tables, which would make it easier to see the differences.

Answer: Thank you for the suggestion which was kindly accepted in order to make visualisation of data easier for the reader. We changed table 1 to a different type of figure (pie chart), now figure 1.

Referee 3 (Reviewer: Grazia Palomba)

Thank you very much for accepting to comment on our work.

Comment 1: KRAS point mutations are highly prevalent in CRC and mostly occur in codons 12, 13, and 61. The profile of KRAS mutations was analyzed by direct sequencing of KRAS codons 12 and 13 only. Although not frequently mutated in CRC (5-6% of cases), codon 61 should have been analyzed (in this large patients’ collection, more than 400 cases could have been misclassified).

Answer: The reasons for not analysing codon 61 are already discussed in the answers to review 1.

Comment 2: More information about the distribution of mutations, according to additional clinico-pathological characteristics of CRC patients, should be presented [mainly, anatomical site of primary tumor (right, transverse, left colon, etc.), disease stage, tumor grading].

Answer: The criteria to take part in the study were that the patients should be at disease stage IV (metastatic colorectal cancer). Information such as anatomical site of primary tumor were not essential to take part into the programme, therefore we cannot include this information in the article.

Comment 3: The discussion about the importance of sexual hormones in the risk for CRC should be more concise. Previous studies have indeed investigated sex-related differences in the prognostic impact of KRAS and BRAF mutation in CRC. No significant association was found between KRAS mutations and sex. Further studies directly focused on the association of hormonal factors with KRAS mutation status in CRC are awaited.

Answer: We have already added the references of other studies that have investigated sex-related differences, which are discussed on page 11-12 (discussion section). We mentioned that we have the largest cohort showing differences between the male and female ratio in Brazil. Actually in China, two different studies performed similar analyses (excluding BRAF) and they also observed a female over male prevalence in KRAS mutant cases. Their cohorts were much smaller than ours and the discussion is written in the discussion. We definitely agree that further studies are needed to clarify associations of hormonal factors with KRAS mutation status in CRC.
Comment 4: why did the authors use the semi-nested PCR for the mutational analysis?

We used semi-nested PCR due to the quality of our samples, which were derived from paraffin-embedded tumour block (formalin-fixed) coming from different Brazilian regions. Usually, DNA derived from FFPE samples can be highly degraded and cross-linked due to fixation. We found some amplification problems using direct PCR, therefore the problem was solved by making a second round of amplification using semi-nested.

Comment 5: In the first paragraph of the Results, the last sentence should be removed (it has been repeated twice the fact that a part of samples was not analyzed).

Answer: Thank you for observing the mistake, the last sentence was removed as shown in paragraph 1 of the results section.

In order to conclude our answers to the reviewers we would like to mention that we believe our results will be of significance to a wide scientific and clinical audience since this KRAS analysis was the biggest performed on a colorectal cohort in our country. We have shown that different variables could be associated with KRAS mutational status. However, we agree that further experiments are necessary to understand these differences and to increase the knowledge on KRAS biology and its association with new therapies, early detection, and disease monitoring.

Thank you very much for reviewing our work.

Sincerely,
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