Author's response to reviews

Title: Different metastatic pattern according to the KRAS mutational status and site-specific discordance of KRAS status in patients with colorectal cancer

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Author's response to reviews: see over
Dear Editor

Thank you very much for reviewing carefully and kindly about our manuscript and getting the opportunity to improve our manuscript. We revised our manuscript based on the referee's comments. We have addressed the reviewers' comments point-by-point in the cover letter (below) and the changes in the manuscript are identified using a red font. We appreciate the reviewers again for their considerate and scientific suggestions. At this time, we hope that our manuscript would be suitable for publication at *BMC Cancer*.

Thank you very much again and look forward to hearing from you in the near future.

With warmest regards,

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Reviewer 1: Ramon RZ Salazar

3 Major compulsory revisions: 4 questions

1. Add p value in the analysis where there seems to be higher relapse rate on the few metachronic cases in mutant KRAS, irrespective of which the numbers are too small to claim a clinically significant and relevant difference

2. Same for the analysis where it appears that Metastasis sites appear more frequent in WT when synchronic, which does not make sense and is subsequent to the higher percentage of KRAS wild type colorectal cancer

⇒ We have performed additional analyses for your 1st and 2nd comments and have added new sentences accordingly. We have also added these contents to Table 5 and 6. To avoid lengthy table, initial metastatic sites ≤ 3 cases were described only in the manuscript.

(1) Pages 9-10: KRAS mutational status and metastatic patterns in Results, the 3rd – 4th sentences

(2) Pages 28-29: Tables 5 and 6 were also revised

3. Please revise discussion accordingly to these previous remarks 1 and 2.

⇒ Based on our answers about remarks 1 and 2, we have revised the Discussion section of this manuscript.

(1) Page 13: Discussion, the 4th sentence in the 3rd paragraph

(2) Page 13: Discussion, the 7th sentence in the 3rd paragraph

4. Finally in the last paragraph of the discussion section in my opinion the recommendation on guiding surveillance and biopsing lung metastases, albeit only suggested, is stil too strong: data are not so robust and need to be further independently validated. Regarding lung mets biopsies, these are not without serious risk of complications, therefore I would strongly suggest to avoid such recommendation before other independent data adds to the current evidence, and in any case, a discussion/exploration on more sensitive assays on the
As pointed out by the reviewer, we have revised some sentences in the Discussion section of this manuscript.

(1) Page 16: Discussion, the 3rd – 4th sentences in the 6th paragraph
(2) Page 16: Discussion, the 7th sentence in the 6th paragraph

Reviewer 2: Oliver Sieber

Major Compulsory Revision

1. Of the 12 and 13 KRAS MT cases that had discordant primary and metastasis mutation status, what proportion of each group were in the recurrent group vs the stage IV group. Or in other words, given that KRAS MT was generally associated with lung metastasis, was there a greater number of discordant cases showing a gain of mutation in lung metastases than the other way round? To answer this question Table 3 should be stratified by whether cases were initially stage IV vs. patients that recurred.

We thank for your kind and thoughtful comments. According to your opinion, we have performed additional analyses in KRAS discordant cases (N = 25). We have analyzed the difference in discordance pattern according to (1) clinical presentation (stage IV or recurred) at the time of initial diagnosis of MRCRC and (2) metastatic sites from which tissue specimens were obtained. There were no statistically significant differences in discordance pattern with respect to these characteristics. Especially, lung metastasis showed no significant difference in discordance pattern compared with other paired metastatic sites. Small sample size might be contributed to this finding. We have added these contents to the revised manuscript and made Table 4.

(1) Pages 8-9: Concordance of KRAS status in primary tumors and related metastases in Results, the 3rd – 4th sentences
(2) Pages 14-15: Discussion, the 10th – 12th sentences in the 4th paragraph
(3) Page 27: Table 4 was newly added in the revised manuscript.
Minor Essential Revisions

# Abstract (pg3)

2. KRAS results are presented both for concordance and discordance between lung metastases and primaries. Perhaps this repetition could be avoided.

⇒ Following the reviewer’s comment, sentences in the abstract was a bit modified

(1) Page 3; lines 18-21

3. The conclusion of abstract has a grammatical error with the use of “concordance”.

⇒ We are really sorry for finding no grammatical error with the use of “concordance” in conclusion of abstract. We have received English proofreading again for this revised manuscript and thus ask for your understanding.

# Background

4. Well written except for the end of second to last paragraph that could be slightly re-worded

⇒ We read the ‘background’ section carefully again and again. However, we could not find the reiterated part. The second paragraph describes the differences between KRAS MT and WT tumors. The third paragraph describes the possible mechanisms or hypotheses on the refractoriness of some KRAS WT tumors to anti-EGFR therapy. If the reviewer kindly points out the reiterated part, we will consider further revision accordingly.

# Methods

5. Re-sequencing of mutations was not performed for confirmation – this should be considered.

⇒ We agree with you about the necessity of re-examination of discordant cases. We discussed this point with the pathologist (HS Lee), but re-sequencing was not feasible in
all cases because of insufficient remaining paraffin tissues for further examination in some cases. Another point to be considered is that repeating the exactly same procedure (Sanger method) is not thought to change the results because the sequencing analysis is very commonly conducted and familiar test at our institution. Confirming the discordance by more sensitive methods such as PCR may be more reasonable. However, PCR was not conducted in our study, partly because tissue specimens were insufficient for further tests in some cases and partly because authors considered that this discordant rate reflects real clinical situation. As mentioned in the Discussion, sequencing (Sanger) analysis is the most frequent method used in the real clinical practice setting. More sensitive methods, such as real-time PCR for KRAS mutation analysis, are only used in the investigational setting and not widely spread in the clinical practice. We thank the reviewer again for considerate and rational suggestion, but ask for the reviewer’s understanding of authors’ situation.

- We have added this content to the revised manuscript.

(1) Page 15: the 5th sentence of the 5th paragraph in the Discussion section

6. Should the word “individual” in line three of stats section be plural?

➔ As pointed out, we have changed from individual to the pleural form.

(1) Page 7: individual → individuals. (Statistical analysis in Methods, the 1st sentence; the 3rd line)

# Results

7. Did not mention the Gly13Cys mutation in the text i.e. numbers of mutations in text add up to 74 instead of 75 as presented in table 2

➔ We omitted the KRAS mutation types with low frequency (i.e. Gln61Leu, Gly13Cys, Gln6His, and Gln61Arg) in the body of the previous manuscript. Following your comment, we have added the mention on these mutation types in the revised manuscript.

(1) Page 8: Frequency and types of KRAS mutation in Results, the 4th sentence
As pointed out, we have revised the manuscript to use a series of normal brackets inside square brackets according to your comment. We have found the same mistakes in other sentences, and corrected the sentences under the same rule.

(1) Page 3: Results in Abstract, last sentence

(2) Page 10: KRAS mutational status and metastatic patterns in Results, the 1st sentence in the 3rd paragraph

(3) Page 10: KRAS mutational status and metastatic patterns in Results, the 3rd sentence in the 3rd paragraph

(4) Page 11: Discordance rates of KRAS status according to the respective metastatic sites in Results, the 4th sentence in the 1st paragraph

# Discussion

9. Difference in discordance from biopsy DNA analyses vs. resected sample DNA analysis is worry (mentioned at top of pg 15). Can the authors provide further evidence that this did not affect the conclusions?

We authors understand the reviewer’s concern. In our study, small number of primary tumor specimens included biopsied specimens (10/143), not resected specimens. Although biopsied specimens of primary tumors showed a trend for higher discordance rate than resected specimens (40.0% vs. 15.8%; P = 0.073), multivariate analysis including this variable did not verify statistical significance in this study. When biopsied specimens are used for a KRAS mutational analysis, it is important to increase the percentage of viable tumor cells to increase sensitivity of sequencing method. We used tumor cell enrichment by microdissection under the supervision of experienced pathologists. In addition, biopsy of primary tumors was all performed by endoscopic biopsy. Actually, a needle biopsy of distant metastasis can be more problematic than endoscopic biopsy of primary tumors in context of tumor cell percentage. In the present study, small number of metastatic specimens (8/143) was obtained from needle biopsy
and only 1 case had KRAS discordance [1/8 (12.5%) for biopsied metastatic specimens vs. 24/135 (17.8%) for resected metastatic specimens; P = 1.000]. These findings suggest that high discordance rate in lung metastasis was not simply caused by types of tissue specimens (biopsied vs. resected). We had described these contents and added new sentences to the Discussion section of the manuscript.

(1) Pages 15-16: Discussion, the 10th – 14th sentences in the 5th paragraph