Author’s response to reviews

Title: Accurate Detection of KRAS, NRAS and BRAF Mutations in Metastatic Colorectal Cancers by Bridged Nucleic Acid-Clamp Real-Time PCR

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Author’s response to reviews:

Dr. Matteo Pasini
Editor
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Dear Dr. Pasini,

Please find attached a revised version of our manuscript “Accurate Detection of KRAS, NRAS and BRAF Mutations in Metastatic Colorectal Cancers by Bridged Nucleic Acid-Clamp Real-Time PCR,” (Manuscript Number: MGNM-D-19-00188R1), which we would like to submit for publication as a Research Article in BMC Medical Genomics.

We would like to thank the editors and reviewers for their quite constructive and helpful comments. Following the reviewers’ advice, we revised the manuscript. We hope that this revision makes our manuscript suitable for publication in BMC Medical Genomics. The modified sections are highlighted in red in the revised manuscript.

Yours sincerely,

Yosuke Hirotsu, PhD.
Responses to the comments of Reviewer #1
I proposed a serial dilution experiment for determination of the sensitivity of the BNA clamp assay. The authors do not show any data that such experiments have been performed. Therefore, I do not find the work suitable for publication unless data from such experiments is provided in a convincing and scientifically acceptable manner.

>Response<
Thank you for your comment. I would like to provide the dilution experiment data using the BNA-clamp PCR in Supplemental Figure 3. The data showed the observed limit of sensitivity was approximately 1-2%. I wrote additional information about dilution experiment in the sections of METHOD (page 9-10, line 162-167), DISCUSSION (page 14, line 236-237) and Figure Legends (page 25, line 515-520).

Responses to the comments of Reviewer #3
1. According to Supplemental Figure 1, BNA-probe clamps wild-type allele. On the other hand, the authors mentioned "BNA-probe designed with TaqMan probe" in the page 7. The role of BNA-probe is inconsistent in the figure and the text.

>Response<
Thank you for pointing out a mistake. I deleted “designed with TaqMan probe” in revised manuscript.

2. The authors set the threshold line at 0.04 in order to decide whether mutations exist. Then results of some samples that amplification plot did not reach to threshold line by the BNA-clamp PCR should be determined mutation negative.

>Response<
If the PCR amplification is visually confirmed but not reaches threshold line, there are possibility that low amount of mutation is exist in tumor sample according to the manufacture’s protocol. Therefore, we try to detect these low amount mutations by Sanger sequencing using the PCR product from BNA-clamp PCR. After performing Sanger sequencing, we assessed whether the mutation was positive or negative.

3. The limit of detection of the BNA-clamp PCR was 1-5%. In sample #3, although the VAF of KRAS A146T and NRAS G12D by the NGS were 28.3% and 9.1% respectively, amplification plot of these did not reach to threshold line using the BNA-clamp PCR. The authors need to describe the explanation in the manuscript.

>Response<
Thank you for constructible comment. Although the precise reason is not unclear, there are several possibilities. One is the quality of the DNA from formalin-fixed embedded (FFPE) tissue. FFPE DNA was fragmented and may be not effectively amplified by BNA-clamp PCR in this sample. Second possibility may be the difference of DNA polymerase enzyme used for reaction. In general, high-activity and fidelity DNA polymerase is used in NGS library construction. The PCR amplification efficiency may be different between BNA-clamp PCR and NGS. According to these possibilities, NGS could detect the mutation nevertheless less quality
of DNA was used as long as targeted regions was successfully amplified and NGS library was construct. These possibilities may lead to the BNA-clamp reach the threshold line, even though the tumor had the more than 5% detected by NGS. I described these possibilities in revised manuscript in DISCUSSION (page 16, line 277-286)