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

Authors:

Yuki Nagakubo (nagakubo-bfjc@ych.pref.yamanashi.jp)

Yosuke Hirotsu (hirotsu-bdyu@ych.pref.yamanashi.jp)

Kenji Amemiya (amemiya-bdec@ych.pref.yamanashi.jp)

Toshio Oyama (t-oyama@ych.pref.yamanashi.jp)

Hitoshi Mochizuki (h-mochiduki2a@ych.pref.yamanashi.jp)

Masao Omata (m-omata0901@ych.pref.yamanashi.jp)

Version: 1 Date: 02 Aug 2019

Author’s response to reviews:

Dr. Matteo Pasini
Editor
BMC Medical Genomics

2 August 2019

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-19-00188), 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

The authors claimed that the BNA-oligos based qPCR methods is slightly superior. I do not think this is a correct conclusion as they explained they used DNA from different sections and therefore it would be hard to make such a conclusion with not so many samples tested. Therefore, I think that a more general comparison would be to test the performance of the two assays using serial dilution of DNA carrying a mutation of interest. They can use clinical sample DNA or DNA extracted from a cell line with known mutation. By doing such a comparison they would be able to determine the sensitivity of the two assays and draw a more reliable conclusion.

Response

Thank you for your helpful comments. As your comments, the direct comparison between two methods could not be assessed because the different sections were tested in this study. I completely agree with your idea that I cannot conclude which method was superior. However, driver mutations such as KRAS and NRAS would expand clonally in colorectal cancer. These driver mutations possibly exist even in different tumor slices prepared from the same tumor tissue. To demonstrate the BNA-clamp PCR accurately detect mutations, I subjected all 50 samples to NGS analysis and observed complete concordant results between BNA-clamp PCR and NGS. Therefore, BNA-clamp PCR method accurately detected KRAS and NRAS mutations.

According to manufacturer’s instructions, the limit of detections of both BNA-clamp PCR and PCR-rSSO methods were 1-5%. The performance of sensitivity is comparable. According to reviewer’s suggestion, we made serial dilution of DNA and performed BNA-clamp analysis. The result showed that limit of detection by BNA-clamp PCR was approximately 1-3%. I described these information in revised manuscript (page 13, line 228-230).

Besides, it is well known that liquid biopsies are expected to gain wider implication in diagnosis and follow-up of solid tumors. If one of the methods is sensitive enough it could be used to detect mutations in cell free DNA from CRC patients. It is advisable that the authors discuss this option as well. The manuscript is written in professional English language and in a logical way. The methods are described in sufficient details.

Response

Thank you for constructive comment. I discussed about cell free DNA in DISCUSSION as follow:
Furthermore, circulating tumor DNA (ctDNA) was shed into the blood stream and body fluids, called as liquid biopsy. The detection of ctDNA is useful for monitoring tumor recurrence, predicting treatment effect and detecting drug-resistant mutation in patients with colorectal cancer. BNA-clamp PCR would be one of the candidate methods for detecting rare mutation in liquid biopsy in a clinical laboratory (page 16, line 273-277).

Responses to the comments of Reviewer #2

1. The title of this manuscript is "Rapid Detection ....", but the content didn't sufficiently show the details of rate. The title or content should be modified.

Response

Thank you for helpful comments. As your suggestions, the title did not represent our data. I changed “Rapid” to “Accurate” in the title. Modified full title is as follow:
“Accurate Detection of KRAS, NRAS and BRAF Mutations in Metastatic Colorectal Cancers by Bridged Nucleic Acid-Clamp Real-Time PCR” (page 1, line 1).
2. In the page 3, the authors mentioned, "high sensitivity and accuracy" in the conclusion section of the abstract; however, any sensitivity in the text cannot be found.

Response
As your comment, this is misleading. I deleted the word “sensitivity and” in this sentence.

3. In the page 5, the second line describes PCR-rSSO. The authors should justify why the method is legitimate.

Response
In Japan, PCR-rSSO assay is approved for in vitro diagnostic test. I inserted the explanation in this sentence as follow:
(PCR-rSSO) method, which is approved for in vitro diagnostic test for analyzing KRAS, NRAS and BRAF in patients with colorectal cancer in Japan (page 5, line 76-77)

4. In the page 5, BNA-clamp PCR is mentioned. An example of BNA-clamp PCR should be added, or if Figure 1 is the example, the details of the conditions and Ct values should be enumerated.

Response
According to reviewer’s comment, Ct values were denoted in Supplemental Table 1. Some samples showed visible amplification, but did not reach to threshold line. Even though these samples, PCR products by BNA-clamp PCR were subjected to Sanger sequencing. As a result, we identified somatic mutations in these samples.

We also described the condition of BNA-clamp PCR in METHODS as follow:
The threshold line was set at 0.04. The threshold cycle (Ct) value was assigned to each PCR reaction and amplification curve was visually assessed. When amplification plot did not reach to threshold line, we examined whether the sample harbored mutations by Sanger sequencing. (page 7, line 123-126)

5. In the page 6, the meaning of "94% positive and 95% negative agreement" is not clear.

Response
Thank you for pointing out ambiguous sentence. We modified as follow:
Positive percent agreement was 94% (29/31) and negative percent agreement was 95% (18/19). (page 10, line 178-179)

6. In the page 6, it would be clearer if the authors details the meaning of "98% on target sites and an average of 11, 768-fold coverage depth".

Response
I explained more detail as follow. Because I performed NGS using additional samples, the data is changed:
NGS yielded the sufficient sequencing reads mapped on the target regions (mean: 97%) and an average base coverage depth on targeted reference region (mean: 10,849-fold) (page 11, line 189-191)

7. In the page 7, BRAF mutation detections were described, but there are no data shown in the manuscript. A table or a figure can be added with adequate description.
I added the data about BRAF mutation data in Table 1.

8. In the page 8, the authors mentioned "PCR-rSSO takes 4.5 hours per run" and "BNA-clamp PCR takes only 2 hours per run" in the Discussion section. These observations could belong to the Results section and describe more.

I described the turnaround time in Result section. I modified as follow:

Turnaround time
We assessed the turnaround time of PCR-rSSO and BNA-clamp PCR methods. It takes approximately 4.5 hours with PCR-rSSO, whereas 2 hours with BNA-clamp PCR. Because PCR-rSSO method uses DNA-probe for hybridization to detect mutated DNA, it takes more long time for hybridize reaction. Contrary, BNA-clamp method contains mixed BNA-clamping probe and primers in reaction reagent and needs one-step PCR reaction for detecting mutation. (page 12-13, line 213-219)

9. In the page 9, for the further visualization, an Ion Reporter Genomic Viewer was used. The authors should show the data in the Supplement data section.

Thank you for your comment. I visualized data on Ion Reporter Genomic Viewer and inserted Supplemental Figure 2. I revised the Figure legends as follow:
Supplemental Figure 2. Sequence reads were visualized by Ion Reporter Genome Viewer. Representative images of read alignments (BAM files) of sample #2 were visualized with Ion Reporter Genome Viewer. There are no mutated reads corresponding to NRAS p.G12C (c.34G<T: chr1:115,258,748) in the next generation sequencing data. (page 24, line 492-496)

10. In the page 11, the conclusion is insufficient.

As your suggestion, I added the conclusion as follow:
In this study, we estimated the performance of BNA-clamp PCR method to detecting KRAS, NRAS and BRAF mutations in colorectal cancers and compared the results from PCR-rSSO and NGS. BNA-clamp PCR accurately detected KRAS, NRAS and BRAF mutations in patients with colorectal cancer. BNA-clamp PCR need one-step PCR for detecting mutations and its turnaround time was approximately 2 hours. Genetic testing by BNA-clamp PCR has potential to be used in routine clinical practice for the selection of appropriate patients for anti-EGFR therapy. (page 16, line 280-286)

11. in the page 13, the method real time RCR was used, but it is not clear whether SYBR green or TaqMan was used. More description would be needed.

TaqMan Probe was used in BNA-clamp PCR method. I added the description in Method section as follow:
This kit contains nine types of BNA-probe designed with TaqMan probe (page 7, line 112)

12. In Table 1, at least one NGS results obtained from both PCR-rSSO and BNA-clamp PCR should be shown. For example, #4 or a higher number of the samples should be checked.
I analyzed the additional samples and obtained results of all 50 samples in this study. The results showed the data of remaining samples were concordant. The data was denoted in Table 1. I also revised manuscript as follow:
as well as 47 concordant samples (#4-#50) (page 11, line 188). Furthermore, remaining 47 samples were concordant among PCR-rSSO, BNA-clamp PCR and NGS. (page 11, line 197-198).

13. Table 2 should include the meaning of Mapped reads, on target, Mean depth, and Uniformity.

I described the abbreviation in Table 2 as follow:
Mapped reads, number of sequencing reads that were mapped to the human genome.
On target, percentage of mapped reads that were aligned over the target region.
Mean depth, Average base coverage depth over all bases targeted in the reference.
Uniformity, percentage of target bases covered by at least 0.2x the average base read depth.

Responses to the comments of Reviewer #3
The authors evaluated BNA-clamp PCR method by detection of KRAS, NRAS and BRAF mutaions in colorectal cancer and comparing with PCR-rSSO method. The overall concordance rate between the two methods was 94% and the BNA-clamp PCR results of three discrepancy between the two methods were consistent with the next generation sequencing results. BNA-clamp PCR method has several advantages compared with PCR-rSSO method. However, the manuscript is not meet requirement of Submission Guidelines in BMC Medical Genomics because the abstract dosen't include Methods section. There is no evidence shown that the BNA-clamp PCR has high sensitivity and accuracy for the detection of BRAF mutations in colorectal cancer. Therefore I cannot recommend the paper for publication in the journal.

Thank you for your comments. According to Editor’s suggestion, I used the Journal Transfer Service from BMC genomics without any modification of manuscript. Therefore, I regret to say that the entire article structure does not meet the Submission Guidelines in BMC Medical Genomics in current version. As your comment, we modified the structure of Abstract (page 2, line 25-30) as well as overall manuscript (List of abbreviations, page 17, line 288-301). A revised version would meet the requirement of Submission Guideline.

We subjected the samples harboring BRAF mutation to NGS analysis. We observed concordant results of BNA-clamp PCR and those of NGS for detecting BRAF mutation. Because I did not show the detection sensitivity, I deleted “sensitivity” in this sentence.