PEER REVIEW HISTORY

BMJ Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form (**http://bmjopen.bmj.com/site/about/resources/checklist.pdf**) and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below.

This paper was submitted to a another journal from BMJ but declined for publication following peer review. The authors addressed the reviewers' comments and submitted the revised paper to BMJ Open. The paper was subsequently accepted for publication at BMJ Open.

ARTICLE DETAILS

TITLE (PROVISIONAL)	Validation of targeted next-generation sequencing for RAS mutation detection in FFPE colorectal cancer tissues: comparison with Sanger sequencing and ARMS-Scorpion real-time PCR
AUTHORS	Gao, Jie; Wu, Huanwen; Wang, Li; Zhang, Hui; Duan, Huanli; Lu, Junliang; Liang, Zhiyong

VERSION 1 - REVIEW

REVIEWER	Troncone, Giancarlo University of Naplese Federico II, Scienze Biomorfologiche e Funzionali
REVIEW RETURNED	28-Jun-2015

GENERAL COMMENTS	This is a nice study, methodologically well performed and well written. I believe that this paper is interesting for the pathological audiences. I have three main suggestion: 1) I would focus the paper not only on exon 2 KRAS, but on the overall RAS. The authors demonstrate that beyond KRAS exon 2 there are other additional 10/51 (19%) cases that are ont elegible for anti-EGFR treatment. This cases were always confirmed by Sanger. Then, which is the point to focus on exon 2 KRAS only? In the context of current guidelines, the Therascreen is not longer a suitable option, then I would not focus the paper on the comparison between NGA and Therascreen, but I would present all RAS data. 2) In this context, I woul discuss the un-expected high prevalence (11%) of exon 4 KRAS mutation. This is interesting, as the UK guideline (J Clin Pathol. 2014 Sep;67(9):751-7.) do not suggest to test for KRAS exon 4. How the authors explain such high KRAS exon 4 percentage. 3) Ideally, the inclusion of low-mutant samples, would be interesting to underline differences between NGS and Sanger. Please include details on the percentage of neoplastic cells in any examined case. Minor points: Abstract: Please, refer consistently to KRAS exon 2, rather than refering either to RAS (aim) and KRAS exon 2 (conclusions).

	Gene should be, always, be wrtittwn in italics Page 5 of 23; lanes 37-40. Please rfeer to previous studies on the potential clinical application of Ion Torrent PGM for determining KRAS mutation status in FFPE colorectal adenocarcinoma (Malapelle U J Clin Pathol. 2015 Jan;68(1):64-8; Tsongalis GJ Clin Chem Lab Med. 2014 May;52(5):707-14.). Page 6 of 23; please give the range of neoplastic cell percentage; This shoul be added as am additional columm to TABLE 3. Please, state the thicknee of the up to 10 unstained sections FFPE sections. The cases were all surgical? There were biopsies? Pagee 7 of 23. Please clarify how nany patient samples were loaded on the same 314 chip. The 200X coverage was applied also to call wild type cases? Conversely there were RAS type cases with less than 200x in any of the relevant amplicons? Figures: Please show forward rather than reserve sequences. It would be much easier for thr pathologist to understand the presence and the type of mutation.

REVIEWER	Fiorentino, Michelangelo Santa Orsola-Malpighi Hospital, Pathology, Addarii Institute of Oncology I have written in the past papers on the same topic using the same methods, but I disclose any personal interest with the companies producing the instruments and the reagents utilized in the present study.
REVIEW RETURNED	01-Jul-2015

GENERAL COMMENTS	This methodological paper investigates the concordance on the detection of KRAS mutation in 51 colorectal cancer samples among three common methodological approaches (Sanger sequencing, real-time allele- specific PCR and massive parallel sequencing with the Ion Torrent platform. The authors found an excellent concordance between NGS and Sanger sequencing while the only case that turned out discordant with the comparison to real time PCR was due to the pre-designed characteristics of the allele-specific PCR technique. They also found that the Ion Torrent platform is sensitive as much as real time PCR. The paper is pretty straightforward and the results clear. The main weakness of the paper is the lack of novelty. There are several other studies comparing these methods even in larger cohorts of patients, all concluding that the NGS approach with any platform is comparable or even better than real time PCR and Sanger sequencing. The most interesting part of this study would have been the description of the mutational status of the other genes covered by the Ion Torrent cancer panel (p53, BRAF, TP53, APC, MLH1, PIK3CA etc.), but unfortunately these data are not shown in the present manuscript. The description of the results of the entire cancer panel would bring novelty and certainly strengthen the paper.

VERSION 1 – AUTHOR RESPONSE

Reviewer: 1

Comments to the Author

I have three main suggestion:

1) I would focus the paper not only on exon 2 *KRAS*, but on the overall *RAS*. The authors demonstrate that beyond *KRAS* exon 2 there are other additional 10/51 (19%) cases that are ont elegible for anti-EGFR treatment. This cases were always confirmed by Sanger. Then, which is the point to focus on exon 2 *KRAS* only? In the context of current guidelines, the The*RAS*creen is not longer a suitable option, then I would not focus the paper on the comparison between NGA and The*RAS*creen, but I would present all *RAS* data.
Response：
Thank you for your kind suggestion. The therascreen PCR kit was included in the present study for the following reasons. First of all, the Therascreen *KRAS* assay reliably detects *KRAS* exon 2 mutations, and is more sensitive than Sanger sequencing. Moreover, although the Therascreen assay is not longer a suitable option based on the latest guidelines as the reviewer mentioned, it is approved by FDA for *KRAS* mutation detection using DNA extracted from FFPE CRC tissue, and currently commonly used in clinical practice. Therefore, it was used together with Sanger sequencing to validate the sensitivity and specificity of NGS in detecting *KRAS* point mutations in FFPE CRC specimens. In fact, the comparison between NGS and Therascreen was used only in the first part of our Results section, and we have presented all *RAS* data in the last part of our Results. Accordingly, we have focused on not only *KRAS* exon 2 but also *RAS* data beyond *KRAS* exon 2 in the Introduction and Discussion section.

2) In this context, I would discuss the un-expected high prevalence (11%) of exon 4 *KRAS* mutation. This is interesting, as the UK guideline (J Clin Pathol. 2014 Sep;67(9):751-7.) do not suggest to test for *KRAS* exon 4. How the authors explain such high *KRAS* exon 4 percentage.
Response：
This is an interesting and helpful suggestion, and we have added related discussions about this in the Discussion section as follow: "*In previous studies, among RAS mutations beyond KRAS exon 2, mutations in KRAS exon 4 were found to occur most commonly in CRC patients. KRAS exon 4 mutations were identified at amino acid residues K117 and A146, and the overall incidence of KRAS exon 4 mutations in patients with KRAS exon 2 wild-type tumors varied from 3.7 to 9.3%.Given that KRAS codon 117 mutation was not evaluated in some studies, the actual incidence of KRAS exon 4 mutations might be underestimated . We also found exon 4 KRAS mutations at K117 and A146 (19 A146T, 1 A146V, and 3 K117N) in our 51 cases at a relatively high frequency (11.8%, 6/51). This should be regarded with caution due to our small sample size, and warrants further exploration in larger studies.*"

3) Ideally, the inclusion of low-mutant samples, would be interesting to underline differences between NGS and Sanger. Please include details on the percentage of neoplastic cells in any examined case.
Response：
Thank you for your kind suggestion. We have included details on the percentage of neoplastic cells in our cases in the METHODS section: "*Tumor cell content ranged from 60% to 95%, with a median cellularity of 80%.*"And tumor cell percentage has been added as an additional column to TABLE 3 according to your suggestion. As for low-mutant samples, the lowest *RAS* mutation frequency detected was 14.3% in our 51 samples, which made Sanger sequencing technically difficult to detect as shown in Figure 6 and the third part of the Results section. We also discussed this as follow in the last paragraph of the Discussion section: "*As demonstrated in our study, identification of variants that occur at a low frequency in Sanger sequencing chromatograms is especially difficult.*"
To further compare the sensitivities of Ion Torrent PGM and Sanger sequencing for low-frequency mutations detection, we performed a dilution series experiment as described in the second part of the Results section. Our results showed that Ion Torrent PGM sequencing was able to detect the *KRAS* mutation (c.35G>A) at a level of as low as 1%, whereas the Sanger sequencing data were difficult to interpret when the dilutions fell below 10%. Similar results were obtained with TP53 mutation (c.818G>A).

We have also added discussion of the limitations in the last paragraph of the Discussion section "*Given that few cases had low-frequency mutations in our study, larger study are warranted to further assess the clinical utility of Ion Torrent PGM in detecting low-frequency mutations.*"

Minor points:

Abstract: Please, refer consistently to *KRAS* exon 2, rather than refering either to *RAS* (aim) and *KRAS* exon 2 (conclusions).
Response：
Thank you for pointing that out. We have made revision according to your suggestion.

Gene should be, always, be wrtitten in italics
Response：
We have made revision according to your suggestion.

Page 5 of 23; lanes 37-40. Please rfeer to previous studies on the potential clinical application of Ion Torrent PGM for determining *KRAS* mutation status in FFPE colorectal adenocarcinoma (Malapelle U J Clin Pathol. 2015 Jan;68(1):64-8; Tsongalis GJ Clin Chem Lab Med. 2014 May;52(5):707-14.).
Response：
Thank you for your kind suggestion. The studies mentioned have been cited in the revised manuscript.

Page 6 of 23; please give the range of neoplastic cell percentage; This should be added as an additional columm to TABLE 3.
Response：
According to your kind suggestion, we have added tumor cell percentage as an additional column to TABLE 3.

Please, state the thickness of the up to 10 unstained sections FFPE sections. The cases were all surgical? There were biopsies?
Response：
According to your kind suggestion, we have added "*(5 mm in thickness)*"and "*(36 surgical specimens and 15 biopsy specimens)*" in the Methods section.

Pagee 7 of 23. Please clarify how many patient samples were loaded on the same 314 chip. The 200X coverage was applied also to call wild type cases? Conversely there were *RAS* type cases with less than 200x in any of the relevant amplicons?
Response:
Two patient samples were loaded on the same 314 chip. A target base coverage of 200X was also applied to call wild type cases, and no cases were found with a coverage of less than 200x in the relevant amplicons of *KRAS*/*NRAS* exons 2,3 and 4.

Figures: Please show forward rather than reserve sequences. It would be much easier for the pathologist to understand the presence and the type of mutation.
Response：
Thank you for your kind suggestion. We have shown forward sequences for Sanger sequencing in Figure 3. However, we preferred reserve sequences for Sanger sequencing in Figure 1,4 and 5,because it was used for a straightforward comparison with NGS sequences displayed by IGV. To make it easy to understand, we have added "*(reverse sequencing)*"or "*(forward sequencing)*"after "*Sanger sequencing*" in the figure legends. If you still feel necessary, we are ready to make further revision.

Reviewer: 2
Comments to the Author
This methodological paper investigates the concordance on the detection of *KRAS* mutation in 51 colorectal cancer samples among three common methodological approaches (Sanger sequencing, real-time allele- specific PCR and massive parallel sequencing with the Ion Torrent platform.

The authors found an excellent concordance between NGS and Sanger sequencing while the only case that turned out discordant with the comparison to real time PCR was due to the pre-designed characteristics of the allele-specific PCR technique. They also found that the Ion Torrent platform is sensitive as much as real time PCR.
The paper is pretty straightforward and the results clear.
The main weakness of the paper is the lack of novelty. There are several other studies comparing these methods even in larger cohorts of patients, all concluding that the NGS approach with any platform is comparable or even better than real time PCR and Sanger sequencing.
The most interesting part of this study would have been the description of the mutational status of the other genes covered by the Ion Torrent cancer panel (*BRAF, TP53, APC, MLH1, PIK3CA* etc.), but unfortunately these data are not shown in the revised manuscript.
The description of the results of the entire cancer panel would bring novelty and certainly strengthen the paper.
Response:
Thank you for your kind suggestion. As we know, the recently updated NCCN Guideline strongly recommends genotyping of tumor tissue (either primary tumor or metastasis) in all patients with metastatic colorectal cancer for *RAS* (*KRAS* exon 2 and non- exon 2, and *NRAS*) , and patients with any known *KRAS* or *NRAS* mutation should not be treated with cetuximab or panitumumab. Given the sample-, cost- and time- inefficiency of traditional methods, NGS platform has great potential for clinical application in performing such multiplex genetic testing in FFPE CRC specimens. Although there are several other studies comparing NGS platform and other methods as mentioned, the potential clinical application of Ion Torrent PGM for determining *RAS* mutation status in FFPE CRC samples has not yet been well investigated, which is especially meaningful based on the latest NCCN colonrectal cancer guidelines. Therefore, we made a comparison of Ion Torrent PGM with two commonly used and widely accepted methods in clinical practice (traditional Sanger sequencing and the Therascreen assay) in the present study.
Our present manuscript was mainly focused on the validation of targeted NGS in *RAS* mutations detection in FFPE CRC specimens. Discussions on other genetic alterations identified by our NGS panel might therefore be out of the scope of the current study. Based on results of the present study, we have performed targeted NGS in another 120 FFPE CRC specimens. The mutational status of the other genes covered by the Ion Torrent cancer panel (*BRAF, TP53, APC, MLH1, PIK3CA* etc.) in these cases will be described in detail in another manuscript to avoid focus shift. If you still feel necessary, we are happy to provide additional information of the other genes in our 51 cases.

VERSION 2 – REVIEW

REVIEWER	Michelangelo Fiorentino Laboratory of Oncologic Molecular Pathology S.Orsola-Malpighi Hospital Bologna 40138 Italy Molecular Pathology Genito-Urinary Pathology
REVIEW RETURNED	12-Sep-2015

GENERAL COMMENTS	As regards the comments raised by this reviewer on the lack of novelty of the study the Authors answered that the novelty is warranted by the new NCCN guidelines on the molecular characterization of CRC for predictive purposes. I'm still convinced that the main advancement that this paper could bring is the analysis by NGS of the molecular alterations involving genes other than the RAS family. Unfortunately the authors answered that they have these results but they want to dedicate a different paper to these findings. As a matter of fact, if the molecular characterization of CRC would

	remain just limited to the ALLRAS and BRAF analyses there would be no need of NGS since other simple and less time consuming methods are avaialble. The implementation of NGS in CRC rather comes from the actual need to expand the number of molecular analyses to other gene alterations.

REVIEWER	Giancarlo Troncone Department of Public Health, University Federico II, Naples, Italy
REVIEW RETURNED	27-Sep-2015

GENERAL COMMENTS	My previous concerns had been nicely addressed. This is a nice study, methodologically well performed and well written. I believe that this paper is interesting for the pathological audiences.

VERSION 2 – AUTHOR RESPONSE

Reviewer: 1

As regards the comments raised by this reviewer on the lack of novelty of the study the Authors answered that the novelty is warranted by the new NCCN guidelines on the molecular characterization of CRC for predictive purposes.

I'm still convinced that the main advancement that this paper could bring is the analysis by NGS of the molecular alterations involving genes other than the RAS family. Unfortunately the authors answered that they have these results but they want to dedicate a different paper to these findings.

As a matter of fact, if the molecular characterization of CRC would remain just limited to the ALLRAS and BRAF analyses there would be no need of NGS since other simple and less time consuming methods are avaialble. The implementation of NGS in CRC rather comes from the actual need to expand the number of molecular analyses to other gene alterations.

Response：

Thank you for your kind suggestion. We have added a supplementary figure to describe the details of the molecular alterations involving genes other than the RAS family according to your suggestion. (Please refer to the third part of our Results section and the supplementary Figure S1) Given the main focus and the limited space, we have not adequately discussed it in this manuscript.

As for all RAS and BRAF mutation analyses, according to our own experience, although several methods are available, they were either insensitive (for example, Sanger sequencing) or cost-consuming and limited to specific mutations (for example, ARMS and other PCR-based assays) compared with PGM and thus were not very suitable to simultaneously detect all RAS mutations (including all KRAS and NRAS exon 2, 3, and 4 mutations) and BRAF mutations (also including mutations other than V600E) in the context of current guidelines as another reviewer mentioned before. Moreover, even only used for RAS and BRAF mutation analyses, Ion Torrent PGM is also sample-saving and time-effective in comparison with other tradition methods. RAS and BRAF mutation analyses can be performed in a single test and the sequencing and data processing can be finished within 24 hours.