Author's response to reviews

Title: Mismatch repair and treatment resistance in ovarian cancer

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Author's response to reviews: see over
Concerning Manuscript revision (ID: 2015462169965480)

Dear Editor,

Herewith we submit the revised version of our manuscript entitled “MISMATCH REPAIR AND TREATMENT RESISTANCE IN OVARIAN CANCER” by Helleman et al. for consideration for publication in BMC Cancer (ID: 2015462169965480)

Thank you for reviewing our manuscript. We have carefully considered all comments of both referees and utilized them for the revision of the manuscript. Our detailed reply on the comments and the revised parts are enclosed on separate pages.

We hope you will judge our paper of sufficient interest to justify publication in the BMC Cancer.

We remain, on behalf of the co-authors,

Sincerely,

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Attachement: Detailed response to reviewer comments (4 pages)
MISMATCH REPAIR AND TREATMENT RESISTANCE IN OVARIAN CANCER
by Helleman et al. (MS: 2015462169965480)

Detailed response (R) to comments (C) of Reviewer 1

C: Reviewer 1 felt that the wrong markers were used for the determination of MSI in ovarian cancer: as stated
“The authors use two mononucleotide markers BAT25 and BAT26 and they didn't found any tumor with MSI. This result can be explained because they use wrong locus for ovarian cancer, I suggest to use other panel of microsatellite locus in order to clarify their result. These microsatellites (BAT25 and BAT26) belong to the Bethesda panel for colorectal cancer”.

C: A similar point was raised by reviewer 2:
“The adequacy of Methods in determining MSI is questionable. The NCI consensus markers were not wholly studied ... Only the latter two markers were analyzed. Another common marker for MSI, NM23, was not studied either. Therefore, the declaration that no MSI and no alterations in MMR were found is not acceptable”.

R: Reviewer 1 and reviewer 2 their concern was that we have only studied 2 markers, i.e. BAT25 and BAT26, in our original paper, which are supposively not specific for ovarian cancer. We slightly disagree, since the so-called colorectal panel but specifically the BAT25 and BAT26 markers, are the most widely used markers for MSI in different types of cancer including ovarian cancer.

The referee may have overlooked this but we have summarized in the Discussion section (page 9-10) and in Table 2 (page 18) all studies that determined the frequency of MSI in ovarian cancer. The two markers BAT25 and BAT26 have been used in 10 of the 14 studies performed since the introduction of the NCI colorectal panel in 1998 (Boland et al. Cancer Res 1998). Only eight authors used all five markers simultaneously (Table 2, page 18).

To meet the referee’s concern we have studied and included two additional markers, i.e. BAT40 and D2S123, as shown in the Materials and Methods section (page 5-6, the added sentences are in bold):
“..Microsatellite analysis was standard performed in our laboratory as described by Westenend et al. (40) using the two mononucleotide markers, BAT25 and BAT26. In addition, the 75 ovarian carcinomas were also analyzed with the mononucleotide marker BAT40 (n=42) or with the dinucleotide marker D2S123 (n=40). So all ovarian carcinomas were analyzed with three or four MSI markers.”

The results obtained with these additional markers have been included in the Results section (page 7):
“..All other cell lines showed no aberrations. In addition, the 75 ovarian carcinoma tissues and the four normal stromal controls showed no aberrations for BAT25, BAT26 and BAT40 or D2S123, indicating that these are microsatellite stable (MSS).”

In the Discussion section we have included that based on these markers, BAT25, BAT26, BAT40 and D2S123, no MSI was detected (page 9):
“.In contrast, the abundant methylation seen in the remaining carcinoma was associated with the lowest MLH1 mRNA expression level of all 50 ovarian carcinomas tested. However, none of the ovarian carcinomas showed MSI for BAT25, BAT26 and for BAT40 or D2S123 which suggests a frequency of MMR inactivation of 0%.”
**C: Reviewer 1 wished to be informed “Why the authors didn’t use the expression of the MMR proteins by IHC?”**

R: Paraffin embedded tissue was not available for all carcinomas. We therefore determined the expression at mRNA level on the frozen samples from our frozen tumor bank since the mRNA’s were available for most of the carcinomas (50 from the 75). Moreover, this is the most direct way to confirm the inactivation of the MLH1 gene due to promoter methylation. However, we obtained the paraffin embedded blocks of 13 patients, including blocks of the six tumors with a low level of MLH1 promoter methylation and the one tumor with a high level of MLH1 promoter methylation. The protein expression of MLH1, MSH2, MSH6 and PMS2 was determined by IHC. We used as an external positive control a colon carcinoma slide, and as an internal positive control the normal stromal cells.

We have observed that some parts of the slides containing tumor and stromal cells were negative while all the tumor and stromal cells of the colon carcinoma control slide were positive for all four genes. This indicates a suboptimal labeling of the ovarian carcinoma slides most likely due to the old age of the paraffin blocks (originating from 1987-1992). These preliminary data has not been included in the manuscript.

However, we should emphasize that in all 13 ovarian carcinomas tumor and stroma cells were positive for all four MMR genes. This suggests that the observed methylation did not result in complete loss of MLH1 expression, and this could explain the absence of MSI in these retrospectively collected ovarian carcinomas.

**C: Reviewer 1, requested to better explain the relation between methylation and mRNA expression.**

“The author should confirm the low methylation and the tumor with complete methylation by sequencig. In the other hand they need to explain better the relation between methylation and mRNA expression (numbers 1 and 0 in figure 3. B)”.

R. The referee may have overlooked in the Materials and Methods section that the sequence of the promoter site including the transcription binding site is known and has been included in our primer design (page 6):

“We designed and optimized primers that are specific for methylated and unmethylated CpG islands within the MLH1 promoter (....). Both primers are located within a region important for a maximal transcription of MLH1 (including the binding site for the transcription factor CBF) (41, 42), since methylation at this region is most likely to inhibit transcription of the gene.”

Moreover, the inactivation of the MLH1 gene due to promoter methylation was confirmed with qRT-PCR (and IHC). The six tumors with low level of MLH1 promoter methylation appeared to express MLH1 at mRNA (and protein) levels similar to that of the unmethylated tumors. Thus a low level of methylation does not result in an altered expression of the MLH1 gene. In contrast, abundant methylation seen in only one of the carcinomas was shown to be associated with the lowest MLH1 mRNA expression level of all 50 ovarian carcinomas tested. However, tumor and stroma cells were positive for the MLH1 protein (IHC) and no MSI was detected. This suggests that the abundant methylation did not result in complete loss of MLH1 expression and the observed low MLH1 mRNA (and protein) expression might be sufficient enough for a functional MMR.

To better explain the relation between methylation and mRNA expression we added the following sentences to the Discussion section (Page 9):

“...Furthermore, we analyzed MMR status in 75 ovarian carcinomas to determine the frequency of MMR inactivation in ovarian cancer in vivo. Seven of the 75 ovarian carcinomas showed MLH1 promoter methylation. We confirmed whether the observed MLH1 promoter methylation results in the inactivation of the gene by determining the MLH1 mRNA expression with quantitative RT-PCR. The six tumors with low level MLH1 promoter methylation appeared to express MLH1 at mRNA levels similar to that of the unmethylated tumors. Thus a low level of methylation does not result in an altered expression of the MLH1 gene. In contrast, the abundant methylation seen in the remaining carcinoma was associated with the lowest MLH1 mRNA expression level of all 50 ovarian carcinomas tested. However, none of the ovarian carcinomas showed MSI for BAT25, BAT26 and for BAT40 or D2S123 which suggests a frequency of MMR inactivation of 0%. The low MLH1 mRNA expression seen in the abundant methylated carcinoma might be sufficient enough for a functional MMR which results in the observed absence of MSI.”
Detailed response to comments of Reviewer 2:

C: Reviewer 2 states that “the declaration that no MSI and no alterations in MMR were found is not acceptable” since only two markers of the five NCI consensus markers were used.

R: A similar comment was made by reviewer 1. Although in our experience and that of Gras et al. (Cancer 2001) (see Discussion page 10: “Gras et al. suggest that the reliability of the mononucleotide markers BAT25 and BAT26 is so high that most MSI can be predicted by evaluating these two markers exclusively (27), confirming the less stringent role for the various markers used for the analysis.”)

most of the MSI cases are detected by using only the BAT25 and BAT26 markers. We, however, agree with the referee that some MSI cases might be missed. This could for example have been the case for the one tumor sample showing abundant methylation of MLH1 promoter which was associated with the lowest mRNA expression level. However, as mentioned above in the response to the second and third comment of reviewer 1, MLH1 mRNA and protein expression was detected, which suggests that the observed methylation did not result in complete loss of MLH1 expression. Moreover, 40 of the 75 ovarian carcinomas were also tested with the NCI marker D2S123 (including the tumor showing abundant methylation and four tumors showing a low level of methylation). All 40 tumors showed no MSI. In addition, 42 of the 75 ovarian carcinomas were also tested and appeared all stable for BAT40, including the two remaining ovarian carcinomas with a low level of methylation. So all ovarian carcinomas were analyzed with three or four MSI markers which decreases the change of missing an MSI case and the MLH1 methylation observed was not related to any of these MSI markers. This has been explained in the reply to reviewer 1 as well.

To meet the reviewer’s comment we have included the data that we obtained for the BAT40 and D2S123 markers in the manuscript in the Material and Methods section (page 5-6): “...Microsatellite analysis was standard performed in our laboratory as described by Westenend et al. (40) using the two mononucleotide markers, BAT25 and BAT26. In addition, the 75 ovarian carcinomas were also analyzed with the mononucleotide marker BAT40 (n=42) or with the dinucleotide marker D2S123 (n=40). So all ovarian carcinomas were analyzed with three or four MSI markers.”

In the Results section (page 7): “...All other cell lines showed no aberrations. In addition, the 75 ovarian carcinoma tissues and the four normal stromal controls showed no aberrations for BAT25, BAT26 and BAT40 or D2S123 indicating that these carcinoma tissues are microsatellite stable (MSS).”

And in the Discussion (page 9): “…Furthermore, we analyzed MMR status in 75 ovarian carcinomas to determine the frequency of MMR inactivation in ovarian cancer in vivo. Seven of the 75 ovarian carcinomas showed MLH1 promoter methylation. We confirmed whether the observed MLH1 promoter methylation results in the inactivation of the gene by determining the MLH1 mRNA expression with quantitative RT-PCR. The six tumors with low level MLH1 promoter methylation appeared to express MLH1 at mRNA levels similar to that of the unmethylated tumors. Thus a low level of methylation does not result in an altered expression of the MLH1 gene. In contrast, the abundant methylation seen in the remaining carcinoma was associated with the lowest MLH1 mRNA expression level of all 50 ovarian carcinomas tested. However, none of the ovarian carcinomas showed MSI for BAT25, BAT26 and for BAT40 or D2S123 which suggests a frequency of MMR inactivation of 0%. The low MLH1 mRNA expression seen in the abundant methylated carcinoma might be sufficient enough for a functional MMR which results in the observed absence of MSI.”
C: Reviewer 2, Please explain why in your patient population only 46 of 75 patients received platinum-based chemotherapy.

R: Some patients received no chemotherapy (n=11, mainly those with early FIGO stage) or different therapies like alkeran chemotherapy (n=2) or radiotherapy (n=3), for 13 patients the data was not available. The remaining 46 patients did receive platinum-based chemotherapy.