Author's response to reviews

Title: HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression

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Author's response to reviews: see over
Reviewer 1:

In primary tumors few mutations in the APM components have been described. Therefore, the analysis of the type and number of the mutations that the authors have found is very important. However, in the tables showed in the paper the exact location of the frameshift mutations in the exons is not indicated. The authors must specify the mutations found.

We agree with the reviewer that this information is vital. Hence have introduced a new table (Table 4b) describing the somatic mutations found in the RST cases (among which mutations in all APM members). We present the position of the repeat in the gene transcript and the number of nucleotides that are inserted or deleted.

Minor essential revisions: All the minor revisions suggested by the reviewer were adopted in this new version including a table specifying all primer pairs used in this study and a more detailed description of the flow cytometry-related procedure.

Reviewer 2:

However there is one major concern:
The authors have analysed three different tumor cohorts:
1. MSI-H HNPCC patients
2. right sided MSI-H
3. right sided MSS
This discrimination of tumor cohorts is not clearly visible in the manuscript.

There are three cohorts in the abstract
There are two arrays in the methods section. There is no discrimination in numbers for the sporadic right sided ones concerning MSI-status. Just reading the text, they are all MSI-H, which was confirmed by methylation analysis. What is needed here:
· Confirmation of the HNPCC cohort. Are all tumors caused by germline mutations or is there just a positive family history with an MSI-H tumor. For a study like this, I would ask for proven pathogenic germline mutations.
· The RST-cohort includes two sub-cohorts: Patients with MSI-H tumors and patients with MSS tumors.

Have all MSI-H tumors been analysed for hypermethylation of the MLH1 promoter? Did they all have a loss of MLH1 expression? These facts should be stated in the methods section. One can find out in table 1 that the RST-cohort includes 48 MSS tumors and 33 MSI-H tumors, this is not stated in the methods or results section. This missing information abolishes a clear structure of the papers and therefore needs to be added prior to a further review process.

1. Accordingly to the reviewer’s comments we have now clarified along the manuscript that we are dealing with 3 sub-cohorts:

Excerpt (Methods – Patient material and tissue microarrays):

One array, previously described [25], included colorectal tumor specimens from 129 suspected HNPCC patients with MSI-H colon tumors (...) The second tissue array included 3 tumor tissue cores from 81 sporadic right-sided colon cancer (...) Approximately 60% (n=48) of these cases were classified as MSS while the remaining (n=33) possessed a MSI-H phenotype.
2. In order to be assured that all the HNPCC cases are in fact hereditary we have slightly reduced this cohort to 75 cases so that we were able to justify all the cases that we have included in this cohort. We now describe our criteria for integration of cases in the HNPCC cohort:

Excerpt (Methods – Patient material and tissue microarrays):

One array, previously described [25], included colorectal tumor specimens from 129 suspected HNPCC patients with MSI-H colon tumors of which 75 cases were analyzed in the present study after confirmation of their HNPCC status: 73.3% (n = 55) of the latter possessed a germline pathogenic mutation in hMLH1 (n=24), hMSH2 (n=18), hMSH6 (n=12) or PMS2 (n=1), the remaining were MSI-H, without methylation of the hMLH1 promoter and/or with immunohistochemical loss of the MSH2/MSH6 heterodimer and/or possessed a very young age at diagnosis (< 50 yrs old). All cases possessed a family positive history for MSI-H tumors.

Furthermore it is interesting to note that after removal of some of the suspected HNPCC cases the numbers (in terms of percentages) of alterations or immunohistochemical defects have remained the same which indicate that we were already working with a consistent HNPCC cohort.

3. We now state that all sporadic MSI-H cases were hypermethylated for hMLH1 promoter and lost expression of the hMLH1/PMS2 heterodimer. We also define how many cases were considered MSI-H and MSS.

Excerpt (Methods – Patient material and tissue microarrays):

Approximately 60% (n=48) of these cases were classified as MSS while the remaining (n=33) possessed a MSI-H phenotype. The microsatellite instability status of the tumors was determined according to recommendations of the National Cancer Institute/ICG-HNPCC [15]. Moreover all MSI-H sporadic cases have lost the expression of the MLH1/PMS2 heterodimer as assessed by immunohistochemistry. The sporadic status of the MSI-H right-sided tumors (RST) was confirmed by methylation analysis of the hMLH1 promoter using a methylation-specific MLPA assay as previously described [26]. All MSI-H sporadic cases presented with hypermethylation at the hMLH1 promoter.

Minor essential revisions: All minor revisions suggested by the reviewer were introduced in the manuscript

Reviewer 3:

This is an interesting design and sufficient numbers of samples were investigated. Authors investigated the relationship between HLA class I expression and MSI status in colorectal cancers. It has been reported that BRAF mutation and hMLH1 promoter methylation was positively associated with MSI. Thus, it will be very interesting to further investigate BRAF mutation (exons 11 and 15) and hMLH1 methylation in this study. The different genetic or epigenetic patterns may be shown in sporadic MSI-H colorectal cancers and HNPCC. It is also interesting that BRAF mutation and hMLH1 methylation patterns might be associated with HLA class I expression.
1. Despite the very interesting suggestion by the reviewer to relate the \textit{BRAF} mutations status with HLA expression we could not find any correlation between the two. \textit{BRAF} (V600E) mutation analysis was present in about 40\% of the MSI-H RST tumors as described in the literature and generally absent in the MSS tumors and HNPCC. However it is distributed equally between tumors that lost vs. retained expression of HLA class I.

Excerpt (Discussion):

\textit{We separately analyzed the presence of the characteristic \textit{BRAF} V600E somatic mutations in the RST cohort (data not shown). Forty-percent of MSI-H sporadic tumors presented with this mutation which was absent in the MSS tumors. It was previously described that this mutation is also absent in HNPCC tumors [44]. V600E was distributed equally between tumors that lost vs. retained expression of HLA class I in the sporadic MSI-H cases.}

2. \textit{hMLH1} promoter methylation was used to confirm the sporadic nature of the MSI-H tumors included in the RST cohort and we found that all the tumors possessed hypermethylation of the \textit{hMLH1} gene promoter.

Excerpt (Material and tissue microarrays):

\textit{The sporadic status of the MSI-H right-sided tumors (RST) was confirmed by methylation analysis of the \textit{hMLH1} promoter using a methylation-specific MLPA assay as previously described [26]. All MSI-H sporadic cases presented with hypermethylation at the \textit{hMLH1} promoter.}