**Author's response to reviews**

**Title:** Colorectal carcinomas in MUTYH-associated display histopathological similarities to microsatellite unstable carcinomas

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**Version:** 2 **Date:** 4 May 2009

**Author's response to reviews:** see over
Dear editor,

Please find below our point-by-point response to the concerns of the reviewers.

Yours sincerely,

Maartje Nielsen

Reviewer's report
Title: MUTYH-associated colorectal carcinomas display histopathological similarities to microsatellite unstable carcinomas
Version: 1 Date: 22 March 2009
Reviewer: Reinhard Buettner

Reviewer's report:
This is a very interesting and well performed study addressing histopathological and molecular features of MUTYH-associated colorectal carcinomas. I think it has strong merits and compiles enough cases (58 carcinoma cases) to validate the conclusions if the mutational data can be completed.

However some of the mutational data were done on significantly smaller numbers and therefore represent only subsets.

The following numbers of cases are given in the text (p 8)
MSI analysis: 42 cases
APC: 36 cases
K-RAS: 38 cases
CTNNB1: 37 cases
p53 immunostaining: 36 cases
p53 sequence analysis: 16 cases
SMAD staining: 26 cases

Since all tumours were used for MMR-protein immunostaining there is no reason why the immunostaining of the other analytes cannot be performed completely.
Also microsatellite analysis works nicely on paraffin-embedded tissue blocks and, hence, should be doable.

Since the main conclusions on the manuscript depend on the percentage of mutations, my strong advice is to perform all of these analyses on all 58 carcinomas listed in table 1. If this is not possible for some reason, only those carcinomas should be listed that were analyzed entirely.

Answers:
We agree with the reviewer. Unfortunately in some of cases no tissue was available or the quality of the DNA only allowed for the less complex (short fragmented) KRAS2 analysis. Also, other studies failed to present complete (staining) data for all the analysed carcinomas (see for example Lipton et al, Carcinogenesis in MYH-Associated Polyposis Follows a Distinct Genetic Pathway, Cancer Research 63, 7595-7599, November 15, 2003).

We agree with the reviewer that it is best to leave out cases with no data or very incomplete data. So, we have removed cases from table 3 in which no analysis was done at all. Also cases in which only MSI and/or KRAS2 analysis was done (and no immunostaining and/or mutation analysis for other genes) were removed, in total seven cases (tumour nr 9,10,15,19,45,52 and 53). Only in one case (tumour nr 14) no data on CTNNB1 or P53 staining are available. Since mutation analysis for APC, KRAS and P53 was successful, we did not exclude this case.
After re-evaluation (with the remarks of the reviewer in mind), we favour to leave out the results of the SMAD4 staining all together. This was decided since in nine cases results were not interpretable because of the lack of proper internal controls in these cases. Under abstract, methods, results and discussion text concerning SMAD4 staining was removed:

- Specifically in the Discussion, page 13, the following sentences were removed: Reduced SMAD4 staining, possibly indicative of a truncated SMAD4 protein or haploinsufficiency provoked by LOH at chromosome 18q, in MUTYH carcinomas was somewhat comparable to that reported in sporadic carcinomas (40% versus 23%). Somehow, staining results for SMAD4, the presence of somatic mutations, and LOH of chromosome 18q results [13] (data not shown) were not concordant. In a recent survey we performed of sporadic rectal carcinomas, a clear relation between LOH status and SMAD4 staining results was found.[42] One explanation might be that all SMAD4 mutations in this study involved missense mutations, and this could lead to a dysfunctional, but not truncated, SMAD4 protein, which possibly can still accumulate in the nucleus. In MSI-high and Lynch CRCs altered SMAD4 staining and mutations are a rare event (Table 2A and B).

Information in Tables: In table 2A and B, the extended version of table 2 and table 3 results on SMAD4 staining from this study and literature were removed.

- In the Abstract under Methods we changed the following sentence to get the numbers more clear (in red the changes):

  From 44 MAP patients who developed ≥1 CRCs, 42 of 58 tumours were analyzed histologically and 35 immunohistochemically for p53 and beta-catenin (encoded by CTNNB1). Cell densities of CD3, CD8, CD57, and granzyme B positive tumour infiltrating cells were determined. KRAS2, the mutation cluster region (MCR) of APC, p53, and SMAD4 were analyzed for somatic mutations.

- In the abstract, results and discussion section the new numbers were changed accordingly. Changed numbers are shown in red in the new manuscript.

**Version:** 1  
**Date:** 24 March 2009

**Reviewer:** Darryl Shibata

**Reviewer’s report:**

Major Compulsory Revisions

The paper by Nielsen et al studied a large group (N=38) of unique colorectal cancers---MAP associated cancers. These cancers are relatively rare, and therefore the details of their characteristics are of great interest. The primary finding is that MAP cancers have characteristics similar to MSI-H CRC. This is interesting because both MSI-H and MAP tumours have underlying defects in DNA repair (MMR for MSI, and BER for MAP).

The primary weakness of the manuscript is the somewhat confused nature of its presentation. It would be helpful to have a Table in the manuscript (rather than the SOM) that summarizes in a simple manner the major differences/similarities between MSI-H, MAP, and CIN tumours with respect to a few critical clinical parameters (such as average age, stage distribution, basic histology, mutation frequencies (APC, KRAS)). I believe such a Table would make it easier for a reader to see that the MAP cancers more resemble MSI-H tumours.

We thankfully accepted the valuable suggestion of this reviewer to add a table in order to improve the readability of results (Table 2B).

**Table 2B Concise overview of data in Table 2A**

<table>
<thead>
<tr>
<th></th>
<th>MAP</th>
<th>Sporadic CRC</th>
<th>Sporadic MSI-high</th>
<th>Lynch (based on MMR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average age (years)</td>
<td>49</td>
<td>68</td>
<td>67-75</td>
<td>47</td>
</tr>
<tr>
<td>---------------------</td>
<td>----</td>
<td>----</td>
<td>-------</td>
<td>----</td>
</tr>
<tr>
<td>MAC stage C or D</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Proximal location</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>(Meta) synchronous CRC</td>
<td>+</td>
<td>0</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>Poor Differentiation</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mucinous (&gt;50%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Crohn’s like infiltrate (conspicuous)</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>Necrosis</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>TILs present</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>TILs marked</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>APC mutations</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>KRAS 2 mutations (codon 12/13)</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Beta-catenin (nuclear staining)</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Beta-catenin (CTNNB1) mutations</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P53 (nuclear staining &gt;25%)</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>P53 mutations</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SMAD4 mutations</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>MSI-High</td>
<td>0</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

0 = 0-10%, + = 11-40%, ++ = 41-70%, +++ = >70% ND = no data
* mucinous rate in MAP CRCs in this study was two times more than in sporadic CRCs: 23% and 12% respectively (see also table 2A).

- Table 2 is changed in table 2A and 2B throughout the text
- Results, first sentence, added: at a mean age of 49 years.
- Results under heading literature review, sentence changed: Results are shown in Table 2A and B; different study outcomes were aggregated in Table 2A and Table 2B shows concise conclusions of these data.
- In discussion added in first paragraph: Age at diagnosis of CRC, when compared to sporadic cases, was relatively young in MAP patients and comparable to that in Lynch patients (49 and 47 years, Table 2A and B). MAP CRCs are showed less metastases than sporadic CRCs, but more than in Lynch carcinomas (table 2A and B).

Minor Essential Revisions

Prior studies (by the same authors) have demonstrated that certain MUTYH mutations are associated with different clinical presentations (Gastroenterology Volume 136, Issue 2, February 2009, Pages 471-476). Are there associations of specific MUTYH germline mutations with some of the parameters measured in this new study?

We performed an analysis to find if there is a relation between genotypes as described in our recent article and a number of histopathological parameters. However, we did not find any significant differences between genotype groups with chi square testing. Finding a significant difference might be hampered by the small numbers. We added the following sentence at the end of results (before heading literature review) at page 10:

**No significant geno-phenotype correlations for any of the main histopathological parameters could be found.**
### Table 1: Mutational Cytogenic Changes in MUTYH-CRCs

<table>
<thead>
<tr>
<th>Type of Mutation</th>
<th>Number of Patients</th>
<th>Proportion (N/Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. No truncating mutation</td>
<td>44</td>
<td>2-12</td>
</tr>
<tr>
<td>2. One truncating mutation</td>
<td>7</td>
<td>1-2</td>
</tr>
<tr>
<td>3. Two truncating mutations</td>
<td>2</td>
<td>0-2</td>
</tr>
<tr>
<td>4. G396D/G396D</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>5. G396D/Y179C</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6. Y179C/Y179C</td>
<td>25</td>
<td>3-6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proportion</th>
<th>45% (19/42)</th>
<th>23% (8/35)</th>
<th>9% (3/32)</th>
<th>31% (11/35)</th>
<th>24% (8/33)</th>
<th>17% (6/35)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% (0/6)</td>
<td>20% (1/5)</td>
<td>0% (0/5)</td>
<td>40% (2/5)</td>
<td>17% (1/6)</td>
<td>17% (1/6)</td>
<td></td>
</tr>
<tr>
<td>0% (0/2)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>33% (1/3)</td>
<td>0% (0/2)</td>
<td>0% (0/2)</td>
<td>50% (1/1)</td>
<td>0% (0/2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60% (3/5)</td>
<td>0% (0/5)</td>
<td>0% (0/5)</td>
<td>60% (3/5)</td>
<td>40% (2/5)</td>
<td>20% (1/5)</td>
<td></td>
</tr>
<tr>
<td>41% (9/22)</td>
<td>21% (4/19)</td>
<td>5% (1/19)</td>
<td>28% (5/18)</td>
<td>6% (1/16)</td>
<td>17% (3/18)</td>
<td></td>
</tr>
</tbody>
</table>

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**Discretionary Revisions**

The first sentence of the last paragraph on page 3 is unclear.

To clarify the sentence *(Recently it was reported that MUTYH CRCs are often near-diploid (52%) and commonly contain chromosomal regions of copy neutral loss of heterozygosity (LOH) (71%), we added:*

*In copy-neutral LOH there is no loss of genetic material and this can arise via mitotic recombination, non-disjunction, or deletion and reduplication events. (ref13)*

**Reviewer's report**

**Title:** MUTYH-associated colorectal carcinomas display histopathological similarities to microsatellite unstable carcinomas

**Version:** 1  **Date:** 3 April 2009

**Reviewer:** Milo Frattini

**Reviewer's report:**

The Authors investigated a cohort of MAP patients (with known biallelic germline alterations in MUTYH gene) with several markers, and then matched the results to clinico-pathological parameters. The Authors found a high frequency of CRC arising in the proximal colon, a high prevalence of tumour infiltrating lymphocytes, and a high level of K-Ras mutations (investigated by direct sequencing. For the first two features abovementioned, the Authors concluded that MUTYH CRCs show similarities to CRCs characterized by microsatellite instability.

A careful revision of the paper and of the data must be performed before any definitive evaluation major compulsory comments

- CRCs from MUTYH arise following the Vogelgram, with MUTYH alterations that leads to APC mutations. The authors found a relative low frequency of APC mutations, they investigated only the MCR where at least 60% of APC mutations occur: the authors must revise the tissue used for DNA extraction, and must describe the percentage of tumoural cells in these blocks. alternatively, the authors should investigate the entire exon 15.

We agree with the reviewer that a low percentage of **APC** mutations in this study is remarkable. The percentage of tumour cells in these blocks is indeed important. The H&E blocks were
analysed by two of the authors. In most cases the percentage of tumour cells was above 70% in all cases this percentage was above 50%, which is an acceptable level for reliable molecular pathologic analysis. To inform the reader better we now added at page 5 under heading Molecular analysis the following sentence: The percentage of tumour cells in the areas from which the punches were taken were in all cases above 50% and in most cases above 70%.

- the Authors found an abnormal high frequency of K-Ras mutations (on average 40-50%), almost exclusively characterized by the change c.34G>T. as this type of alterations, although frequent, occur in about 30-40% of K-Ras mutated cases, a contamination of K-Ras mutated allele is possible. the Authors must repeat the DNA extraction in a clean area, without any contamination, and repeat the experiments at least twice.

We indeed agree that finding a high percentage of KRAS2 mutations is remarkable, especially since the vast majority concerns one particular mutation, c.34G>T. With can understand the concerns of the reviewer of the possibility of contamination. However, we have several arguments to assume that contamination is very unlikely. The analyses were done in a CE certified laboratory with high quality standards including analyses of negative and positive controls (not being the c.34G>T tranversion). There are separate laboratory rooms for pre- and post-PCR products. Also a direct PCR and not a nested PCR is used so the stock DNA tube has been opened only once during the first steps of the procedure diminishing the chance of pollution. The same procedure for finding KRAS2 mutations is also performed on a diagnostic basis in this CE certified laboratory. Moreover, our finding is in good agreement with previous reports by others, who reported comparable results (Lipton et al 2005, Jones et al 2004).

- The high frequency of K-Ras mutation, if confirmed, is not shared with CRC characterized by microsatellite instability. the Authors must underline this difference.

Indeed, the high frequency or the sort of KRAS mutation is not shared with Lynch or MSI-high tumours. We changed and added a sentence in discussion, at the end of page 12 and beginning of page 13:

In contrast, KRAS2 mutations are found on average in 29% of sporadic CRCs and 22% of sporadic MSI-high carcinomas. Furthermore, the c.34G>T tranversion comprises just 8% of KRAS2 mutations found in sporadic and none in MSI-high CRCs.[12] In Lynch carcinomas the percentage of KRAS2 mutations is around 34%, Table 2A and 2B) and in these carcinomas other hotspot mutations are found, namely the c.35G>A and c.38G>A, compromising 81% of detected KRAS2 mutations in these tumours. [41]

- As the carcinogenesis followed by MUTYH patients is dramatically different with respect to that followed by CRC with microsatellite instability, the Authors must explain, or at least hypothesize, the observed histopathological similarity.

The observed histopathological similarity between MUTYH and MSI high CRCs focuses on a high rate of mucinous histotype, and increased presence of TILs.

In discussion, page 11, we explain this as follow:

Because of the disruption of the caretaker function of mismatch repair enzymes in MSI-high tumours somatic mutations are accumulated throughout the cell genome. Such abundance of mutations can result in aberrant frameshift peptides that would be presented at the cell surface, through the antigen processing pathway, to cells of the immune system. This sequence of events could explain the presence of an accentuated intra-epithelial lymphocytic infiltrate in MSI-high tumours. In MUTYH carcinomas the disruption of the caretaker function of the BER machinery,
mediated by MUTYH, leads to the accumulation of G>T somatic mutations, at least early in tumourigenesis, which might evoke similar specific anti-tumour immune responses.

We agree with the reviewer that a possible explanation for the found similarities could be more explicitly mentioned, so we added under conclusion the following sentence:

The presence of TILs might suggest that defects in base excision repair, similar to mismatch repair deficiency, produce secondary aberrant proteins functioning as tumour-specific neoantigens that, in turn, induce anti-tumour immune responses.

Other changes made by us:

- Because results MUTYH IHC already has been reported by others with more extensive description of their methods and results than we did, we decided to leave the following sentence out.

  We also performed IHC for MUTYH, but no nuclear staining was seen as reported by Di Gregorio et al (results not shown).[45] Others reported also that MUTYH IHC could not discriminate between MUTYH biallelics and controls [31,46].

- Added under Table 3 in legends: mainly copy neutral LOH and not physical loss.

- Throughout the text, we have replaced "MUTYH CRCs with MAP CRC's. Because the term MUTYH CRCs might be confusing (can also main CRC of MUTYH heterozygote patients).

- We changed the title in Colorectal carcinomas in MUTYH-associated display histopathological similarities to microsatellite unstable carcinomas (previously: MUTYH-associated colorectal carcinomas display histopathological similarities to microsatellite unstable carcinomas)

- Instead of the CTNNB1, the term beta catenin was used in the text.