Author's response to reviews

Title: Mutations in APC, CTNNB1 and K-ras genes and expression of hMLH1 in sporadic colorectal carcinomas from the Netherlands Cohort Study

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Author's response to reviews: see over
Response to reviewer 1 (Dr. Wolf)

With regard to the general comments in the reviewer’s report, we agree with the reviewer that the aims and hypothesis were not explicitly mentioned in the Background, which may also be related to the assumption that the novelty lies mainly in the occurrence of \textit{CTNNB1} mutations. Our main objectives were to study the co-occurrence of alterations in genes involved in the early to intermediate stages of colorectal carcinogenesis and to relate these to tumour and patient characteristics. Accordingly, we have changed the background section of the abstract to “The early to intermediate stages of the majority of colorectal tumours are thought to be driven by aberrations in the Wnt (APC, \textit{CTNNB1}) and Ras (K-ras) pathways. A smaller proportion of cancers shows mismatch repair deficiency. The aim of this study was to analyse the co-occurrence of these genetic alterations in relation to tumour and patient characteristics.” Since we were specifically interested in the early to intermediate stages of colorectal cancer carcinogenesis, \textit{TP53} mutation analysis was not carried out as this is generally considered a late event. We have included a comment on the selection of the cases for \textit{CTNNB1} gene mutation analysis in the Background, to clarify our hypothesis. The statement “mutation analysis of other samples was abandoned since this was deemed irrelevant” refers to selective analysis of \textit{CTNNB1} mutation analysis, and was based on the assumption (derived from reports in literature) that \textit{CTNNB1} mutations are uncommon in tumours with a truncating \textit{APC} mutation in combination with the very low frequency observed in tumours that were expected to harbour \textit{CTNNB1} mutations. Therefore, the statement has been changed to “In addition, mutation analysis of remaining samples was abandoned since this was deemed irrelevant, as these harboured truncating \textit{APC} mutations and are considered to be unlikely to also have \textit{CTNNB1} mutations” (page 10). Please also see response to reviewer 2.

BAT-26 analysis was performed to assess the concordance between hMLH1 expression and the microsatellite instability marker. We drew a random sample of patients for this. As the frequency of hMLH1 defective tumours is low (10 to 15%), and we therefore expected tumours showing this defect to be under-represented in a random sample, we also included 48 tumours lacking hMLH1 expression. We have changed the paragraph on BAT-26 analysis on page 9 to incorporate this explanation.

Response to specific points raised by reviewer 1:

1. The numbers of tumours analysed vary throughout the manuscript. The cause for this variation lies in the success rate of the applied analyses of the available archival tumour tissue samples, and the application of selection criteria. \textit{K-ras} mutation analysis yielded the highest success rate, with 737 tumours analysed. \textit{APC} mutation analysis could be completed for 665 tumours and hMLH1 immunohistochemistry was successful in 724 cases. Because tumours harbouring a truncating \textit{APC} mutation and/or absence of hMLH1 expression were excluded from \textit{CTNNB1} analysis, this analysis was carried out in 464 tumours. In the final number of 656, which is used in the abstract, only those cases were included of which the mutation analysis of both the \textit{K-ras} and \textit{APC} genes as well as the hMLH1 immunohistochemistry was complete. By changing the order of the
paragraphs, and now starting with the most complete analysis, that of the K-ras gene, we hope to have improved the comprehension of the data flow. We have justified the numbers of cases throughout the various sections of the manuscript, with the resulting frequencies now reiterated in the statistical analysis paragraph of the Methods section and in the Results section.

2. We have adjusted the abstract according to the reviewer’s suggestion, and now reads “Mutations at the phosphorylation sites (codons 31, 33, 37 and 45) in the CTNNB1 gene were observed in tumours from only 5/464 patients.”

3. We agree with the reviewer that the information on the questionnaire is superfluous in this context and have therefore changed the paragraph to state the number and age of participants at baseline only.

4. We included the degree of reproducibility to quantify the validity of our data. We consider this to be an important aspect with regard to our results and the conclusions drawn from these, especially as our analyses are performed on archival tissue, which can be difficult due to loss of DNA integrity. Considering the high sensitivity of our methods, we believe that the less than 100% reproducibility reflects a degree of heterogeneity in the tumour rather than inaccurate analysis. We have included a paragraph discussing this matter on page 14, 1th paragraph, “The reproducibility of the applied assays was good, with a reproducibility of 85% and 88% for APC and K-ras, respectively. Arguably, this indicates the extent of heterogeneity present in the tumour samples.”

5. WAVE screening was considered to be more efficient than PCR-RFLP, as aberrations in all four phosphorylation sites can be detected in a single run. In addition, RFLP would require appropriate restriction enzymes. WAVE analysis was optimised and validated using specific mutations in cell line DNA, i.e. HCT116 (codon 45: 3bp deletion) and SW48 (codon 33: C→A) as well as DNA derived from patients’ desmoid tumours (codon 41: A→G and codon 45: C→T) as positive controls. All of these mutations were repeatedly confirmed by sequencing. Moreover, WAVE analysis was carried out at two different temperatures (57.7 en 60°C). We have included this information in the Methods section (page 7).

6. We have included a statement on the use of the Cramér’s V test and the χ² test for analysis of the co-occurrence of K-ras and APC gene mutations in the statistical analysis paragraph of the Methods section (page 9).

7. Paragraphs 2 and 3 on page 10 describe two different analyses presented in tables 3 and 4, respectively. Paragraph 2 describes the differences in patient and tumour characteristics between tumours with and without a defect in single genes (APC, K-ras, hMLH1), whereas paragraph 3 describes the differences in these characteristics between tumours with aberrant APC and/or K-ras and those with defective hMLH1.

8. With regard to reported frequencies of K-ras and APC gene mutations in this study in relation to previously published data, a paragraph on this has now been added to the Discussion and states: “The K-ras mutation frequency of 37% is in accordance with reported frequencies of 30 to 60%. The low frequency of 37% of truncating mutations in the mutation cluster region of APC in this study, however, seems low in comparison to the general assumption that most colorectal tumours
harbour a mutation in the APC gene. When only reports from studies on sporadic rather than familial colorectal cancer or colorectal cancer cell lines are considered, the mutation frequencies are lower and vary between 30 and 70%, and a population-based case-control study in the Netherlands reported a 32% mutation frequency.”

9. Because the mutation cluster region of the APC gene comprises a significant part of the large gene, and we analysed archival DNA, we had to restrict our PCR technique to amplifying several smaller fragments of DNA and designed a PCR protocol for the amplification of four overlapping fragments. Alternative amplification, applying different primers, was used if any of the fragments could not be amplified. A comment on this alternative approach has been included in the APC mutation analysis paragraph of the Methods section. However, despite this, in 72 cases, we were unable to amplify and subsequently analyse one or more of four fragments of the mutation cluster region. To eliminate the chance of false negative results, we excluded any tumour of which one or more of the four fragments could not be analysed successfully. Most likely, the fragmentation of DNA from the archival tissue impaired the analysis. A comment on this, “Tumour DNA was derived from formalin-fixed, paraffin-embedded tumour tissue blocks. Depending on the conditions of fixation and storage, the extracted DNA is more or less fragmented, which may have impaired the analysis of mutations in the APC gene more than in the K-ras gene, since the analysis of the latter is based on the amplification of a smaller gene fragment.” has been included in the Discussion (page 13). Similarly, the quality of archival tissue sections is likely to be the cause of the failure of immunohistochemistry of hMLH1. Please see response to reviewer 2 as well.

10. In the manuscript, activating mutations are defined as missense mutations in codons 12 and 13 of K-ras. In the legend to Figure 1, we have changed the text to “K-ras mutations other than missense mutations in codons 12 and 13 (n=2)”.

11. We have included the $\chi^2$ test and $P$-value in the footnote of table 2.
Response to reviewer 2 (Dr. Salahshor)

1. We have performed alternative PCR amplification using different primer binding sites when amplification of any of the fragments failed at the first instance. Therefore, it is unlikely that mutations in primer binding sites would be responsible for failure of the amplification. Moreover, the amplification of the gene fragments consisted of overlapping fragments. A comment on this alternative strategy has been included in the APC mutation analysis paragraph of the Methods section. A plausible explanation for the lower success rate for the APC amplification compared to the K-ras amplification lies in the size of the amplified area, the amplified domain of K-ras exon 1 is smaller than any of the fragments of the mutation cluster region of the APC gene. Even though a PCR strategy with overlapping fragments was chosen, the chance of unsuccessful amplification of part of the mutation cluster region is greater than in K-ras’ exon 1. A comment on this, “Tumour DNA was derived from formalin-fixed, paraffin-embedded tumour tissue blocks. Depending on the conditions of fixation and storage, the extracted DNA is more or less fragmented, which may have impaired the analysis of mutations in the APC gene more than in the K-ras gene, since the analysis of the latter comprised a smaller segment of the gene.”, has been included in the Discussion (page 12, 13). Our main objective for the APC mutation analysis was to identify the type of mutations rather than limit our analysis to the absence or presence of a truncating mutation. To our knowledge, APC expression to detect defects in APC by immunohistochemistry is not feasible. We have performed immunohistochemistry for β-catenin in a selection of samples, but due to the observed heterogeneity in the samples, the immunohistochemical analysis was deemed inconclusive. Moreover, immunohistochemistry of β-catenin would not provide an appropriate answer to the underlying defect. Please see our response to reviewer 1, too.

2. Previous reports in literature suggested a greater probability of β-catenin mutations in tumours that also showed microsatellite instability, a comment on this has been added to the Introduction. Since microsatellite instability in a large majority is represented by lack of hMLH1 expression harboured a defect in mismatch repair, we considered this worthwhile exploring.

3. For BAT-26 analysis, a random sample of patients was drawn. As the frequency of hMLH1 defective tumours is low (10 to 15%), and we therefore expected tumours showing this defect to be under-represented in a random sample, we also included 48 tumours lacking hMLH1 expression. We have changed the paragraph on BAT-26 analysis on page 9 of the Methods section to incorporate this explanation. Please see the response to reviewer 1 as well.

4. A comment on hMLH1 immunohistochemistry “Nine per cent of tumours showed hMLH1 deficiency, as determined by immunohistochemistry (Figure 2).”, has been added to the Results section (page 11) and two photos showing present (Figure 2A) and absent hMLH1 (Figure 2B) staining have been added.

5. The number of tumours analysed vary throughout the manuscript, because of differences in the success rate of the applied analyses of the available archival tumour tissue samples, and the application of selection criteria. K-ras mutation
analysis yielded the highest success rate with 737 tumours analysed. APC mutation analysis could be completed for 665 tumours and hMLH1 immunohistochemistry was successful in 724 cases. In the final number of 656, which is used in the abstract, only those cases were included of which the mutation analysis of the K-ras and APC genes as well as the hMLH1 immunohistochemistry was complete. Mutation analysis of the CTNNB1 gene was restricted to samples without a truncating APC mutation and/or absence of hMLH1 expression, and therefore this analysis was carried out in 464 tumours. By changing the order of the paragraphs, and now starting with the most complete analysis, that of the K-ras gene, we hope to have improved the comprehension of the data flow. We have justified the numbers of cases throughout the various sections of the manuscript, with the resulting frequencies now reiterated in the statistical analysis paragraph of the Methods section and in the Results section. Please also see our response to reviewer 1.

6. A remark on the absence of alterations of the investigated genes “It is plausible that these tumours have harboured mutations in other components of the Wnt signalling pathway, e.g. mutations in the Axin genes, which are essential for the degradation of β-catenin, and were observed in 11% of patient samples. In addition, in a non-mutational way promotor hypermethylation of APC that leads to impaired APC function has been observed in 18% of sporadic colorectal adenomas and carcinomas.” has been added to the Discussion (page 14, third paragraph).

7. Instead of the reviewer’s useful suggestion for a table that compares the mutation frequencies of our and other studies, a descriptive paragraph including such numbers has been included in the Discussion (page 14).

8. Following the paragraph on the differences in observed mutation frequencies between our study and other reports, a paragraph on the sensitivity and reproducibility of the K-ras and APC mutations detection assays “The method for mutation analysis of the APC mutation cluster region and exon 1 of K-ras is based on nested amplification and direct sequencing of purified PCR fragments, a highly sensitive method. Since no screening step was performed prior to the sequencing of the gene fragments, it is unlikely that mutations would have escaped detection. The reproducibility of the applied assays was good, with a reproducibility of 85% and 88% for APC and K-ras, respectively. Arguably, this indicates the extent of heterogeneity present in the tumour samples.” has been added to the Discussion (page 14, third paragraph). Please see our response to reviewer 1 as well.