Author's response to reviews

Title: High-Resolution Analysis of HLA Class I Alterations in Colorectal Cancer

Authors:

Jan Willem F Dierssen (JWFDierssen@gmail.com)
Noel FCC de Miranda (N.F.Miranda@lumc.nl)
Arend Mulder (A.Mulder@lumc.nl)
Marjo van Puijenbroek (M.van_Puijenbroek@lumc.nl)
Willem Verduyn (W.Verduyn@lumc.nl)
Frans HJ Claas (F.H.J.Claas@lumc.nl)
Cornelis JH van de Velde (C.J.H.van_de_Velde@lumc.nl)
Gert Jan Fleuren (G.J.Fleuren@lumc.nl)
Cees J Cornelisse (C.J.Cornelisse@lumc.nl)
Wim E Corver (W.E.Corver@lumc.nl)
Hans Morreau (J.Morreau@lumc.nl)

Version: 2 Date: 8 September 2006

Author's response to reviews: see over
Dear Dr. Puebla

Hereby we send you our point to point answer to the reviewer’s comments. The suggested alterations were incorporated in the manuscript.

Sincerely,

Hans Morreau

Reviewer 1: Dr. Matthias Kloor

Point 1. A central statement of the paper is that the frequency of HLA class I alterations determined by using FCM in the present study is lower than reported in some previous studies. However, I feel this may be somewhat overstated. There are several potential reasons for this (low number of samples, sample taking issues a.o.) which are independent from the method applied for HLA class I antigen detection. These issues should also be discussed, e.g. the fact that the comparably high number of stage A/B patients (no stage D patients were recruited) may contribute to a lower number of HLA class I alterations.

Answer 1: We now discuss as suggested by the reviewer why the number of HLA alterations differs from previous reports. In addition to the hypothesis that these differences might relate to the sensitivity of the different techniques we have discussed that the composition of the groups of study of the different reports might be the cause of such distinct observations.

Text excerpt (Discussion, 1st paragraph):

Although the discrepancy between our FCM data and the reported IHC data should be verified in a larger series, the discrepancy may be explained by the different technical approaches used and/or by the different composition of the groups under study (e.g. microsatellite stable/MSI-H distribution, tumor staging).

Point 2. Among 21 colorectal cancers, 11 are localized in the proximal colon. Is this the result of a preselection process? This should be discussed.

Answer - 2. There was no preselection of the patients included in this study. We provide now this information in the patient material section of the Material and Methods.

Text excerpt (Material and Methods – Patient material):

There was no pre-selection of the patients included in this series.

Point 3. Paragraph 3 of Results is unclear and should be rewritten. The authors want to emphasize that the four MLH1-negative tumors are most likely sporadic. Instead of referring to "sporadic MSI-H cases" first, and then supporting the assumption, the section should begin with the statement that (1) the respective tumors show MLH1
loss, (2) have no clinical criteria indicative of HNPCC, and are thus most likely sporadic.

Answer 3. We have now converted this stretch into a logical sequence as suggested by the reviewer.

Text excerpt (Results - Alterations of HLA phenotype are found in specific subsets of colorectal cancer, 1st paragraph):

Interestingly, all 5 MSI-H cases in the series (5/21) demonstrated HLA alterations (Table 3). Four of the 5 cases demonstrated loss of HLA-A and –B (p = 0.028); in 1 of the 5 cases, we observed the loss of a single HLA allele (case 191). Loss of expression of the heterodimer of MLH1 and PMS2 was observed in the 4 MSI-H cases using IHC, and was not observed in any other cases (Table 3). These patients do not fulfill any criteria indicative of HNPCC are thus most likely sporadic. Other clinicopathological features typical of sporadic MSI-H tumors [26] were also present; they occurred in elderly patients (mean age 75 years, p = 0.043), 3 of 4 were located in the caecum (p =0.019), and 3 of 4 were peri-diploid (p = 0.053).

Point 4. The conclusive paragraph (Conclusion, paragraph 2) appears to be inappropriate. Vaccination against colorectal cancer is not the scope of the study. Therefore, it is suggested to incorporate the last paragraph in the Discussion section. Moreover, the last sentence should be omitted, because there is still a considerable number of deaths related to sporadic MSI-H colorectal cancer each year which clearly indicate that there is a need for novel therapeutic approaches.

Answer 4. We have deleted the last paragraph of the discussion agreeing with the reviewer that it might be inappropriate for the current manuscript.

Text excerpt (Conclusion):

FCM allows the discrimination of complex phenotypes related to the expression of HLA class I. The different patterns of HLA class I expression might underlie different tumor behavior and influence the success rate of immunotherapy.

Point 5. Reference 8 appears to be wrong. The cited results are from Cabrera et al. 1998, not 1996 (breast cancer).

Answer 5. We have changed the reference.

Point 6. In the text (Material and Methods, Flow Cytometry) it is stated that in 6 cases the HLA phenotype was not known prior to resection. In the corresponding table (Table 2) only 5 cases are marked by asterisks.

Answer 6. There were only 5 cases from which the HLA genotype was not known prior to surgery. We have now corrected this number in the Flow cytometry section of the Material and Methods.

Excerpt (Material and Methods - Flow Cytometry):
In 15 cases, we used a complete panel of human and mouse anti-HLA mAbs, and in another 5 cases we used mouse mAbs (mu-mAb) because the HLA genotype was not known prior to tumor resection.

Discretionary revisions:

In general, I think that the most interesting finding of the paper is the detailed characterization of allele- and locus-specific losses in colorectal cancer and their association with the MSI-H phenotype. Therefore, I would suggest to strengthen this point rather than to focus on the difference between the observed frequency of alterations in the present study and previous ones (Cabrera et al. 1998). Moreover, the first paragraph of the discussion appears to be self-contradictory, emphasizing the higher sensitivity of FCM compared with IHC and the enhanced detection of variations (in addition, the increasing use of FCM is not an argument in that context at all). To my opinion, this would rather argue in favor of a higher frequency of HLA class I alterations observed by this method.

Answer: We agree with the reviewer’s comments and focused in the characterization of the HLA alterations found in this work rather than in the comparison between the results obtained by our work and others.

Reviewer 2: Dr. Barbara Seliger

Point 1: The author state that immunohistochemistry using formalin fixed paraffin-embedded tissues is limited due to the number of antibodies available which could be used on these samples. Although this statement is correct they discuss this issue in the context of work from Cabrera and co-authors who mainly employ fresh frozen tumor samples for analysis. Using this approach a bigger number of antibodies can be implemented for immunohistochemistry and even HLA class I surface expression could be monitored by employing the antibody W6/32.

Answer 1. As suggested by the reviewer we now discuss limitations of both the employment of both paraffin-embedded and fresh frozen material for immunohistochemical analysis. First we state that HLA staining by IHC is often strongly cytoplasmic, which could potentially obscure functionally relevant membranous co-expression and result in a false negative interpretation which is a common problem when working with either fresh frozen or paraffin embedded tissues. We now state that the employment of fresh frozen tissue allows the implementation of a higher number of antibodies although complete panel of antibodies are not yet available. We do not discuss anymore these problems in the light of Cabrera’s et al. work. (Page 4, first paragraph)

Text excerpt (Background – 3rd paragraph):

However, these studies have primarily been based on immunohistochemical analyses (IHC) and therefore have a number of intrinsic limitations [11]. HLA staining by IHC is often strongly cytoplasmic, which could potentially obscure functionally relevant membranous co-expression and result in a false negative interpretation. Additionally, adequate study by IHC is hampered by the limited...
choice of antibodies (Abs) available for the analysis of formalin-fixed, paraffin-embedded tissue and importantly, despite the fact that the use of fresh frozen tissue allows the employment of a higher number of Abs, complete panels of the latter are not yet readily available.

Point 2. Page 6 the sentence _specifically analysed membranous expression of cells for HLA antigen staining_ is not clear.

Answer 2. We have now rephrased the sentence in the Flow cytometry section of the Material and Methods. We wanted to illustrate that the non permeabilization of the cells upon the immunostaining with the anti-HLA antibodies allowed for the measurement of the HLA molecules expressed at the cell membrane alone since the cytoplasm was not accessible to the antibodies.

Text excerpt (Material and Methods – Flow cytometry):

*Importantly, we specifically analyzed membranous expression since cells were only permeabilized after HLA immunostaining making the cytoplasm inaccessible to the anti-HLA Abs.*

Point 3. The low level of HLA-A, B expression might be due to loss or diminished β2-microglobulin expression (#55).

Answer 3. We find loss of HLA A and/or B expression in all the MSI-H cases including case 55. In all of these β2m membranous expression is also diminished but retained. We speculate that the diminished membranous expression of β2m is a consequence of the loss of HLA expression and not the opposite since through IHC β2m staining is still strongly cytoplasmic suggesting that β2m is still being expressed but not presented at cell surface together with the HLA A and B molecules. Furthermore as detected by the W6/32 antibody total HLA expression is still retained suggesting that molecules such as HLA-C and/or HLA non-classical molecules seem to be expressed. For their membranous expression it is essential its association with the β2m molecule. (see also point 6)

Point 4. Table 3 HC does it mean staining with HC-10 or with W6/32? The corresponding sentences in the manuscript have to be congruent.

Answer 4. The staining results under the HC column correspond to the employment of the W6/32 antibody. We have now added this information in the footnote of the table.

Point 5. Page 15 what does high levels of infiltrate mean? Please explain. What is the frequency of CD4+ or CD8+, T cells and NK cells?

Answer 5. According to the reviewer suggestion we have now tried to be clearer in what respects the levels on infiltrate in MSI-H tumors. Menon et al. (Lab Invest 2004) describe that MSI-H tumors are associated with intra-epithelial infiltration of CD8+ and CD57+ cells that is present at much higher levels in MSI-H tumors than in microsatellite stable tumors.
Sporadic MSI-H tumors are usually relatively large, but rarely disseminate \[3,38,39\]. This favorable tumor behavior has been associated with an increased intra-epithelial CD8+ CTLs and CD57+ NK cells infiltrate when compared with the microsatellite stable tumors. CD4+ cells are not frequently found in the intraepithelial infiltrate of MSI-H tumors. \[24,38-42\].

Point 6. Please discuss this. Has \(^{2}m\) been structurally analysed? \(^{2}m\)-microglobulin mutations could not be claimed, since results have to be shown. The extremely low level of \(^{55}\) might suggest structural alterations in \(^{2}m\).

Answer 6. We analyzed the \(^{\beta}2\)m gene for all the MSI-H cases and we did not find any frameshift mutation in the microsatellite sequences of this gene. Kloor et al. (Cancer Res. 2005) have described that mutations in this gene are only found in these specific sequences. According to the reviewer’s request we have included these data in the material and methods and Results section.

Text excerpt:

Material and Methods – Microsatellite instability: \(^{2}m\) frameshift analysis was performed with primers (available upon request) build around coding repeats of the \(^{\beta}2\)m gene; the (CT)4 repeat in exon 1 and two (A)5 repeats in exon 2. \[24\]

Results - Alterations of HLA phenotype are found in specific subsets of colorectal cancer (1st paragraph): Since all the MSI-H tumors that lost HLA A and B expression showed diminished membranous expression of \(^{2}m\) we have screened the microsatellites sequences of the corresponding gene but failed to find any frameshift mutation.

Point 7. The association with microsatellite instability is not convincing since most of the samples have not been analysed for this issues (13/21). Please provide these data.

Answer 7. We have now performed the MSI analysis in the remaining cases from which the MSI status was not known. All of them were microsatellite stable as we inferred from their clinical characteristics and MLH1/PMS2 heterodimer expression. These data were altered in Table 3.