Author's response to reviews

Title: MGMT promoter methylation status and MGMT and CD133 expression as prognostic markers in colorectal adenocarcinoma

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Author’s response to reviews: see over
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Prognostic impact of MGMT promoter methylation and MGMT and CD133 expression in colorectal adenocarcinoma

(previously: MGMT and CD133 protein expression and MGMT promoter methylation status as prognostic markers in colorectal adenocarcinoma)

Reviewer1

These reviewer’s suggestions have been extensive and very useful. We hope that the improvements made in our revised manuscript give answer to the reviewer’s comments.

Minor essential revisions

Comment 1 - Throughout the article, the authors refer MGMT/CD133 expression or MGMT/CD133 staining intensity. It would be more easy to read the manuscript if the authors specify what happens with expression or intensity, for example “higher MGMT expression intensity” or “loss of protein expression”. Several sentences seem to be contradictory because of this.

Response: Following the referee comments and with the aim to facilitate the reading understanding of manuscript, we have specified what happens with molecular expression adding corrections in page/lane: 2/40, 2/42, 9/209-210, 10/231, 11/258-260, 12-13/290-292, 15/351-352). We think that this facilitate the manuscript comprehension.

Comment 2 - In the lines 66-67 of the background, the authors state that Carcinoembryonic antigen has major limitations. It would be important to mention some of them taking in account that new biomarkers are needed.

Response: Following the referee instructions, we have changed this sentence including the main CEA limitations (Page 3, Lane 67-69):“it has major limitations such as low sensitivity and specificity (36% and 87% respectively). In addition, until a rate of 16% may be false positives [5,6]”. In addition, we have included a reference that provides more recent data on the marker CEA in colon cancer (Reference 6: McKeown E, Nelson DW, Johnson EK, Maykel JA, Stojadinovic A, Nissan A, Avital I, Brücher BL, Steele SR: Current Approaches

Comment 3 - In the line 91 of the background, the authors declare "Expression of the two molecules and MGMT methylation status…", I suggest “The expression pattern of the two molecules and MGMT methylation status…”.

Response: We agree with the referee. We have included the change (Page 4, Lane 93-94)

Comment 4 - In the line 147 of the methods section, “all staining were performed” correct to “all staining procedures were performed”, or similar.

Response: We have also included this change (Page 6 Lane 148)

Comment 5 - In the line 157 of the methods section, it is described a detailed score of MGMT staining (-,+, ++, ++++, ++++), however for the analysis the staining is only classified as low and high. Detailed score should be removed. Take this into account also for the Figure 2.

Response: Following the referee recommendations, we have summarized the detailed score (Page 7 Lane 158-160). We also have modified the Figure legend 2 indicating only representative photomicrographs with MGMT staining negative (a), with a <50% (b and c) and with a ≥ 50% (d and e).

Comment 6 - In the line 167 of the methods section, substitute “tumor glands stained for MGMT and CD133 staining independently to dye distribution” by “tumor glands stained for MGMT and CD133 independently to dye distribution”

Response: Following the referee recommendations, we have substituted this sentence (Page7 Lane 166)

Comment 7 - In the results, section Patient characteristics, it is referred that “tumor was instage III in 39.8% of patients”, however in the table is stated 40.2%. The same for the percentage of patients that died due to the colorectal adenocarcinoma. In the table 1, the percentages do not take into account the number of patients without information. Values in text and table should be consistent. In table 1, the unknown values should be presented in the same way as the others, as it is seems like percentage and in fact are (n) values. Data of treatment response should be included in the table 1.

Response: Following the referee recommendations, we have corrected the differences between tables and text (page/lane: 8/186-188,10/229-230, 11/245-249). In addition, we have modified Table 1 (and also the new Table 2- see below) following other more correct ways to represent clinical characteristic data such as those appears recently in this same journal (Tsikitis et al., Predictors of recurrence free survival for patients with stage II and III colon cancer BMC Cancer. 2014; 14: 336). Now, we have included the n(%) indicating the “n” value as a note in table 1 (and 2) facilitating the
comprehension of both tables. Finally, and following the referee instructions we have included the treatment response in table 1.

Comment 8 - The data concerning MGMT promoter methylation status as well as MGMT and CD133 percentage of expression and staining intensity should presented without association analysis in a table or graphically in the respective figure.

Response: Following the referee recommendations, we have made and included a new table titled “Table 2: Molecular characteristics of colon adenocarcinoma patients” with molecular results of MGMT and CD133 without associations and we have updated the numbers of tables in all items. Moreover, we have included the phrase “Table 2 summarizes the molecular characteristics of the patients” in page 9 lane 194 for table introduction and new item (Table 2) (page 10/225)

Comment 9 - Can the authors explain why there are two bands unmethylated in the patients 18 and 20, in the figure 1?

Response: These secondary bands correspond to an unspecific autoannealing band of primers, normally not appear or appear with less intensity. These bands may be observed in several prestigious manuscript such us Esteller M et al. Cancer Res. 1999, 59:793-7.999 and Kim et al. Genes Chromosomes Cancer. 2006, 45:781-9, among others.

Comment 10 - Can the authors comment why it was used bisulfite treatment and methylation-specific PCR instead of bisulfite-sequencing that is more sensitive and give less false-positives results?

Response: We have used bisulfite treatment and methylation-specific PCR because it is a method widely accepted and used by scientist community to obtain an approximation of methylation modulations in the MGMT promoter in paraffin-embedded tumor tissue. Another important reason was that this method is cheaper than bisulfite-sequencing in massive sample processing such as it occurs in our research. Despite this, our next objective is to use bisulfite-sequencing to obtain more specific information about our patient selection.

Comment 11 - In the section “Association of MGMT expression and methylation status with CD133 expression” of the results (line 247), the table referred should be Additional file 1: Table S1.

Response: Following the referee recommendations, we have repaired the error (page 11 lane 245-246)

Comment 12 - The sections “Association of MGMT expression and methylation status with CD133 expression” and “Influence of CD133 expression percentage and MGMT intensity on overall survival and disease-free survival” should be fused into one section.
Response: Following the referee recommendations we have removed the two sections and we have fused both sections in “MGMT and CD133 interactions and clinical influence” section (page 11 lane 243).

Comment 13 - In the section “Influence of CD133 expression percentage and MGMT intensity on overall survival and disease-free survival” of the results, the values described in the lines 253-255 are not the same present in the supplementary table 1.

Response: Following the referee recommendations we have changed these values (page 11, lane 245-249).

Comment 14 - The authors should briefly comment/discuss their results taking in account the main cellular functions of MGMT and CD133.

Response: The cellular CD133 function is still unknown in colorectal cancer. On the other hand, we have exposed the main molecular action mechanism of MGMT in the introduction (page 3-4 lane 72-76). In addition we included the main aspect of MGMT in relation to colon cancer in discussion (page 12 lane 281-289) despite the molecular repairing mechanism of MGMT in CCR is still unknown.

Comment 15 - The scale bars on the immunohistochemistry images should be presented.

Response: We have included the scale bars in immunohistochemistry (figure 2 and 3). We indicate the size in the Figure legends.

Comment 16 - The manuscript needs text editing.

Furthermore, we have detected one error in Additional file 2: Table S2 and we have corrected. We apologize for the error, we have reviewed all the data to avoid any error more.

We have changed the title of table 1 “Table 1 Characteristics of colon adenocarcinoma patients” for Table 1 “Clinical characteristics of colon adenocarcinoma patients”.

We have detected an error in order of appearance in figures and we have substituted Figure 3 for 4 and vice versa.
Prognostic impact of MGMT promoter methylation and MGMT and CD133 expression in colorectal adenocarcinoma

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Reviewer2

We appreciate the comments provided by this reviewer and found them useful for improving our manuscript. We have attempted to address the concerns of reviewer 2 and undertake all suggested modifications.

Comment 1- Personally, I would re-compose the title.

Response: If the main objective of the title is to reflect the importance of MGMT and CD133 for the colon cancer prognosis, it is very difficult to change it. However, following the instructions of the referee, we propose a new title which emphasizes the importance of both MGMT and CD133 as prognostic markers “Prognostic impact of MGMT promoter methylation and MGMT and CD133 expression in colorectal adenocarcinoma”. However, we do not know if this is the idea of the referee.

Comment 2- The authors mention that several patients are already dead. It would be interesting if they can mention how was the level of MGMT expression on these patients.

Response: These data appear as additional information in Table S2. In this table we analyze the interactions between MGMT molecular variables and clinical variables included patient status in last medical examination. Statistical analysis did not show a significant correlation with other variables.

Comment 3- The figures do not have a title that mention what you see there. They should be auto-explanatory. Add please PCR, IHC (and what) and so on.

Response: Following the referee recommendations, we have modified the title of the figure legends.

Comment 4- The association between CD133 and MGMT expression that the authors search for are not strong and a bigger cohort of patients may be needed. Mean while, it would be very interesting to observe if MGMT is expressed in CD133 positive cells/areas in good prognosis/OS/DFS patients and low in CD133 cells for bad prognosis/OS/DFS. I would like to know the results of a co-IHC or IF to have a better understanding of the possible importance of MGMT expression in putative stem cells. All together, the authors add relevant data to the importance of MGMT expression in CRC and its possible role as a disease prognostic marker while CD133 expression and
outcome is more confused. I would suggest the authors to revise what is written here and in a near future add more patient data to conclusively identify if MGMT is a good prognosis marker.

Response: We appreciate the referee suggestions. In this moment, we are interested in sequencing of MGMT promoter to observe changes between patients. Now and with the referee observation, we will consider co-IHC or IF of both biomarkers for future assays which could be included in upcoming publications.