Author’s response to reviews

Title: 1p36 deletion is a marker for tumour dissemination in microsatellite stable stage II-III colon cancer

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Author’s response to reviews: see over
Dear Editor,

Please find the attached revised version of the manuscript entitled: **1p36 deletion is a marker for tumour dissemination in microsatellite stable stage II-III colon cancer**.

We have addressed the comments of reviewer 1 and 2 in a point-by-point response to their concerns (see below). At each point we are referring to where in the manuscript changes have been made. We want to thank the reviewers for the constructive criticism that has improved the manuscript.

The array data has been submitted to the GEO repository and we hope to have an accession number within a few days.

Yours sincerely,

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**Author response to Reviewer 2’s comments**

The paper describes the analysis of somatic copy number alterations (CNA) in a series of 100 plus colorectal cancers by microarrays, comparing cases that did with those that did not disseminate during a 5-year follow-up. The study is interesting and revealed the involvement of a narrow region at 1p36 as predictor for cancer recurrence. The value as prognostic indicator for metastatic spread in colon cancer of this region have been previously reported, but the new study adds some interesting information that is validated by contrasting their data with the data from the Cancer Genome Atlas consortium (TCGA). There are some points that would need to be addressed to improve the clarity of the paper.

A better definition of tumor dissemination needs to be given. In Introduction, it includes metastases present at diagnosis and metastases developed after surgery. This creates confusion that is especially prominent in the Abstract. Thus, the sentence Loss of 1p36.11-21 was associated with tumour dissemination in microsatellite stable tumours of stage II-IV is unclear. Stage III and IV necessarily imply metastases at diagnosis. Also, the sentence of the same section in abstract: “Loss of 1p36.11-21 relative to average copy number of the genome showed similar prognostic value compared to absolute loss of copies, and would apply to more cases” is not completely clear. It should be explained in more detail, like “Loss of 1p36.11-21 relative to average copy number of the genome showed similar prognostic value
compared to absolute loss of copies. Therefore, the use of relative loss as a prognostic marker would benefit more patients by applying also to hyperploid cancer genomes. Space limitations may be the responsible for the lack of clarity, but I guess the word limit may not be strictly endorsed in cases such as this.

Answer: We agree and have now described tumor dissemination as stage IV tumors or recurrent disease in patients diagnosed with stage II or III tumors.
Rows in manuscript: 214-216, 361-367

We have also followed the suggestion by the reviewer and clarified that patients with hyperploid cancer genomes may benefit from using relative loss of 1p36 as a marker.
Rows in manuscript: 64-65

In Background, the sentences Microsatellite stable (MSS) colon cancers have mutations in tumour suppressor genes such as APC and TP53 and chromosomal instability (CIN) resulting in numerous CNAs. Multiple molecular prognostic markers such as high MSI, loss of 18q and reduced 94 SMAD4 expression have been suggested [2–5] should be modified. There is no causal link established between mutations in APC and P53 and CNAs and chromosomal instability has not been shown to occur in all MSS tumors. Also, as the same paper describes in Methods, there is no difference between MSI-L and MSS, therefore there is no need to name it “high” MSI.

Answer: We have now changed the phrasing in the background section to clarify the characteristics of MSS tumors.
Rows in manuscript: 96-98

In addition we specify that low-level MSI is excluded from "MSI", citing the suggested references.
Rows in manuscript: 100

In this regard, the citation given for the lack of difference between MSI-L and MSS (ref. 12, 2013) should include two previous references, Perucho, Cancer Res 1999, and Laiho et al, Cancer Res 2002.

Answer: See above.

Since some of the patients were treated with chemotherapy, the results of the analysis after filtering out the treated patients should be given to test the possible influence of treatment.

Answer: Such an analysis has now been added to Table 2. With all chemotherapy treated cases removed. The resulting association between the deletion and dissemination in SI-III was similar and significant. A higher odds ratio of 9 was observed but the uncertainty was higher as fewer samples were used for the analysis. Removing samples that received treatment did not alter the conclusion that loss of 1p36 is associated to tumor dissemination.
In Methods, the use of >2 and <2 is not very precise in the description of the CNAs:
A) Gain (>2 copies). B) Relative gain (>25% above individual sample average copy number). C) Loss (<2 copies). It would seem that they refer to more than 2 copies over the two already existent in a diploid genome, and the same for <2 that can be taken as the loss of 2 copies. If the value means that anything more than 2 or anything less than 2 are gains and losses, then the issue is the threshold to determine significant gain or loss over the background noise. Is a 1.9 value taken as a loss, or a 2.1 as a gain?

Answer: We have now clarified those sections in Methods and Figure Legends. As integer copy numbers per tumour cell were computed, loss to <2 copies implies 0 or 1 remaining copy etc.

In Table 1 MSI should be also included There is a significant difference bewteen MSI and MSS regarding dissemination (P<0.001). This behavior needs to be discussed in view of the fact that CNAs did not have association with dissemination. As MSI tumors are mostly pseudiploid, and have better outcome than MSS that are aneuploidy, aneuploidy should have a worst prognosis.

Answer: MSI has been included in Table 1 and associates with better prognosis as expected. Although this study contains very few MSI samples with tumour dissemination, we now discuss the indication that presence of CNAs may be associated with dissemination for those patients.

Figure 2 can be eliminated. There is nothing new.

Answer: We agree that Figure 2 does not contain any of the main results in the manuscript. However, we would like it to remain in the manuscript as it motivates some decisions made regarding TCGA MSI/MSS classification. Thus, it is now included as a supplementary figure (Figure S2).

Since Figure 1 and S2 are very similar in the profiles, the fact that no alteration other than the 1p36 loss is taken as a potential prognostic marker is surprising. Perhaps the sentence Out of our primary findings (marked in Figure 1), only deletion of 1p36 was independently identified as a potential prognostic marker (Figure S2), should be modified: identified as statistically significant potential... Also, there is a region of LOH apparently at the same 18p shared by the TCGA and the authors’ data. Is that different region?

Answer:
We have modified the relevant section in Results to clarify the criteria applied in our search for potential markers. Significant association (p<0.05) in the whole (MSS) data set of the current study was required as well as in TCGA (although borderline significance for relative loss was accepted for 1p36 since absolute loss performed very similar with significant association).

*Rows in manuscript: 268-270*

As the reviewer points out, LOH in a small region of 18q is indeed significantly associated with dissemination in both the current study and TCGA data. It was not considered a useful marker due to its lower effect size, but its presence is now properly noted in the manuscript.

*Rows in manuscript: 270-277*

**In Table2, the inclusion of stages II-IV is confusing. What is compared? Cases with and without metastases at diagnosis, or metastases developed at distant organs after surgery? In which case, stage IV should be excluded. Also, the data is not very persuasive, only the comparison including stage IV is statistically significant. What happens if cases IV are excluded?**

**Answer:**
Dissemination of the tumour may have taken place prior to diagnosis despite no sign of metastasis at the time of diagnosis or surgery. With complete resection of the primary tumour, prior dissemination is indeed the most likely explanation for subsequent distant recurrence of stages II-III. Therefore we think it is acceptable to group stage IV with recurrent stage II-III to increase power and improve the estimate of effect size. We have attempted to be more clear about this decision in Results and Discussion.

*Rows in manuscript: 214-216, 361-363*

An analysis of the stage II-III subgroup is now included in Table 2, with significant association between the marker and dissemination. We think it is important to not put too much emphasis on the p-value when looking at smaller subsets (II and III separately). Their effect sizes strongly indicate presence of the association and although not independently significant, higher p-values are expected in such small subsets. We would only doubt the presence of an association within e.g. stage II if we could not see a similar trend.

**Author response to Reviewer 1’s comments**

The study is well designed and technically sound. The authors used the latest technology and bioinformatic tools to approach a genome-wide search for copy number alterations as biomarkers for tumor prognosis at diagnosis. They included colorectal tumors from patients with local disease, with and without recurrence, and metastatic cancers. Authors found a recurrent 15 Mbp deleted region at 1p36.11-21 associated with tumor dissemination. Similar results have been previously reported (References 16-18 in the paper). Thus, the results presented here offer a solid confirmation...
for the prognostic value of 1p36 deletions in colorectal cancer, which is clinically relevant and potentially exploitable.

Major Compulsory Revisions
The reported common deleted region contains at least six target cancer genes but no further analysis is provided. The authors should offer an added value trying to characterize a more precise and specific marker among the target genes to claim for a clinically useful prognostic marker. For example, confirmation experiments by target gene expression from the same cohort using quantitative real time PCR would offer stronger evidences and an affordable analytical system for an extensive use in clinical settings.

Minor Essential Revisions
The last sentence at the Results section (lines 294-296) should be at the Discussion section.

Answer: The last sentence has been moved to the Discussion section.