Author's response to reviews

Title: Comprehensive profiling of DNA methylation in colorectal cancer reveals subgroups with distinct clinicopathological and molecular features

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Version: 2 Date: 25 February 2010

Author's response to reviews: see over
REVIEWER 1

Major Compulsory Revisions

1. In the methods (statistical analysis) or discussion, provide further justification for use of unsupervised hierarchical clustering for their analysis (references from other groups using the Illumina methylation platform and similar analysis methods; results of the authors’ assessment of the robustness of their clustering using non-parametric bootstrap resampling). Why were only the results for unsupervised hierarchical clustering analyses reported, given that supervised hierarchical clustering analyses were performed as mentioned by the authors?

>> Results from supervised analysis were not mentioned in the Results, as they were only used on an ad-hoc basis to explore interesting differences in methylation patterns. An example of this is the supervised analysis based on EMVI status in the Discussion (p16).

>> To clarify the bootstrap analysis, we have added the following text to Methods (p7): “The robustness of clustering was assessed using non-parametric bootstrap resampling analysis. To achieve this we calculated the percentage of times the optimal number of clusters, as determined by BIC, was observed”. In addition, the text “In further support, agreement for the existence of three clusters was X% in bootstrap resampling” has been added to Results (p10).

2. The authors need to acknowledge in the discussion that the three “distinct clusters” they observed may have occurred by chance, given the large number of comparisons and their small CRC sample size. In the results or in additional file 2, they should provide nominal or their Bonferroni-corrected p-values (as the authors allude to in the methods) to support their assertion of three distinct clusters. To this reviewer, a visual inspection of additional file 2 indicates two clearly distinct clusters with a third rather “iffy.”

>> To clarify how we arrived at three clusters, the following text has been added to Methods, (p7): “The optimal number of clusters was determined using Bayesian Information Criterion (BIC)”. Insert p-value in results? Since multiple testing is not used to determine the number of clusters in BIC, no Bonferroni-corrected p-value is available.

>> Nonetheless, we agree that our observation of three CIMP subgroups may be a chance finding and have added the following sentence in the Discussion (p17): “Further investigations in large and independent population-based series are required to validate these findings and to assess the clinical utility of CIMP subgroups.”

3. Given the high correspondence between the authors’ CIMP-H and CIMP-high (Weisenberger panel) in their samples and agreement of CIMP-high and clinicopathological features in other previous studies, the authors should address in their discussion: is the additional expense and complexity of using the Illumina methylation platform potentially beneficial in some regard, given their results appear in large measure to validate the results of smaller methylation panels?
To address this comment, we have added the following text in the Discussion (p16): “The cost effectiveness of using arrays to characterize CIMP-H is questionable, given the strong concordance between CIMP-H from this study and CIMP-w. Further studies should clarify if the additional information provided by methylation arrays is worth the complexity and expense.”

Minor Essential Revisions

1. Methods (statistical analysis), first paragraph, first sentence: “allele” should be plural, as the beta-value is a ratio based on fluorescent signals from methylated and unmethylated alleles at each data point.

>> Allele is now referred to in its plural form.

2. Discussion (p. 13, last paragraph, 3rd sentence should read “..70% of these just one CpG site per gene was evaluated.” rather than “sites.”

>> “CpG sites” has now been revised to “CpG site”

Discretionary Revisions

1. Title may be overly explicit; perhaps remove “three.”

>> The word “three” has now been removed from the title.

2. In the Conclusion paragraph of the abstract, final sentence: revise to “This study provides further confirmation that both KRAS and BRAF mutations are involved with the CIMP-H pathway of CRC.” Previous research groups have suggested and confirmed this as referenced by the authors in the text; thus it is not a new finding, as the current sentence implies.

>> We agree the finding that KRAS and BRAF mutations are associated with CIMP-H is not original. However, some groups have suggested these mutations define distinct CIMP groups. We specifically want to highlight this was not the case in the current array-based study. To clarify this point, the following text has been added to the end of the Abstract: “This study suggests that both KRAS and BRAF mutations are involved with the CIMP-H pathway of CRC rather than with distinct CIMP subgroups”.

3. In the Conclusion paragraph of the abstract, the authors should consider stating that their research appears to validate the smaller sets of methylation markers that others (e.g. Weisenberger) have been running in CRC samples.

>> This was addressed in the original text in the second paragraph of the Discussion (p13) and we feel requires no further comment.

4. Methods, statistical analysis: Either provide more detail (formula) as to how the beta value was calculated, or a reference that provides the formula (e.g. Martin-Subero, et al., PLoS one, 2009). Was background intensity computed from negative controls subtracted from each analytical data point?
We have added the following text to Methods (p7): “Normalisation of background intensity was estimated from a set of built-in negative controls and subtracted from each methylation data point as performed in other studies [28, 30].”

5. Regarding comment 1 under major compulsory revisions, the authors should acknowledge limitations of the unsupervised hierarchical clustering approach and perhaps reference the work of Houseman, et al. (BMC Bioinformatics, 2008) that concludes via simulation analysis of data from the Illumina methylation platform, their recursively-partitioned mixture model is an effective (with respect to classification error) and computationally efficient method for clustering DNA methylation data and is more reliable than other clustering approaches.

We fully acknowledge there may be alternate ways to analyze the methylation data. The statistical method employed here is the most widely used (Bibikova et al; Christensen et al; Ladd-Acosta et al; Nosho et al), thereby achieving a level of standardization between studies.

6. Provide more detail in the statistical analysis as to the determination of the binarized cut-off value of 0.297 for beta-values which was set based on a 5% FDR for the methylated control. Was the FDR based on a simulation analysis, and how many simulations?

To clarify this issue, the text in the Methods (p7) now reads: “Using this threshold, methylated controls in the array were classified as unmethylated at a 5% false discovery rate.” The FDR was not based on simulation analysis.

7. Discussion: Given the relatively high proportion (65%) of CIMP-H tumors in their study especially compared to large population-based studies of CIMP in CRC, does the hospital in which the CRCs were sampled see a certain profile of patient (older, large proportion of smokers, etc.)?

The hospital from which the samples were obtained is a private medical centre with a typical profile of colorectal cancer patients. No obvious bias in patient demographics or clinicopathological features was observed (Table 1). No information was available for smoking status.

8. Discussion (page 12, paragraph 3): the authors state that “the absence of MSI and KRAS and BRAF mutations in the 13 CIMP-M tumors suggests this subgroup may have a distinctive molecular and clinical phenotype.” The authors should also acknowledge that the rates of MSI, BRAF, and KRAS mutation in larger population-based studies of incident CRC (see Slattery, et al. Dis Colon Rectum 2009) would indicate that at most in a sample of 13 “CIMP-M” colon tumors (12 of which are distal), it would be expected that only 1 or 2 tumors would have any of these alterations; the absence of these alterations may have occurred by chance or because they are distal site, and not from an underlying distinct CIMP phenotype.

To address this issue, we have amended the relevant paragraph in the Discussion (p15) to read as follows: “A novel finding of this array-based analysis was the existence of an apparent CIMP-M group (Figure 1). These tumors displayed a higher frequency of EMVI compared to both CIMP-L and CIMP-H, and a significantly
higher stage compared to CIMP-H (Table 1). CIMP-M tumors were almost exclusively located in the distal colon or rectum (12/13, 92%). MSI and KRAS and BRAF mutations were notably absent in these tumors, although this may be due to reportedly lower frequencies of these alterations in distal tumors (SlatteryML’09 DisColonRec52(7):1304-11). Taken together, these results suggest CIMP-M tumors could be a distinct clinical and molecular entity, although confirmation in larger, independent tumor series is required.”

9. Table 1: Regarding distal tumor site, please footnote the numbers of samples that were rectal site (vs. distal colon).

Respective sample numbers have been added as footnote 2 in Table 1 (p24).
REVIEWER 2

Major Compulsory Revisions:

1. Weisenberger et al showed strong association among BRAF mutation, sporadic MSI-high, and CIMP-high (ref 10). In this study, CIMP-high by the criterion of CIMPW also shows strong association to MSI and BRAF mutation. Thus, the criterion of CIMPW is reproducible and easy to follow. However, the CIMP criterion in this study is not easy to follow. Although the authors have categorized 3 CIMP subgroups, the clustering result shows 4 branches (Figure 1). Thus the criterion of CIMP in this study is very obscure, and may be no reproducible. If the other clustering analyzes were used, different results will appear. The authors should address this issue.

>> Please see our response to point 2 of Reviewer 1 Major Compulsory Revisions.

2. In addition, Weisenberger et al showed specific association of KRAS mutation with methylation in several genes amongst their large panel of markers studied (ref 10). Nagasaka et al also demonstrated that KRAS mutation may be associated with an increased level of methylation but the genes affected may be distinct from those associated with BRAF mutation (ref 13 and 16). In this study, CIMP-high is strongly associates with both BRAF and KRAS mutation. Is methylation spectrum of BRAF mutant cancer different from that of KRAS mutant cancer or not?

>> We have added the following paragraph in the Discussion (p15) to address this question: “Since BRAF mutations are strongly associated with CIMP and mutually exclusive to KRAS mutations (Weisenberger; Figure 1), a point of interest is whether methylation patterns differ between tumors with BRAF and KRAS mutations. Supervised clustering analysis with Bonferroni correction revealed that only 1 of the 202 tumor-specific CpG sites was differentially methylated between these tumor groups (HTR1B_P222_F, upregulated in KRAS mutant tumors, \( p = 8.1 \times 10^{-6} \)). HTR1B (5-hydroxytryptamine (serotonin) receptor 1B) is a G protein-coupled multi-pass membrane protein involved in regulation of the serotonin system (Drago et al. Eur J Clin Pharmacol. 2010 Jan;66(1):5-27. Epub 2009 Oct 7). The gene is hypermethylated in lung cancer and its chromosome locus (16q14.1) is frequently deleted in a number of cancer types (Takai D, Oncogene. 2001 Nov 8;20(51):7505-13). However, no links with BRAF or RAS signaling have been reported.

Minor Essential Revisions:

1. Page 9, line 13, sentence beginning "Interestingly, the two patients with CIMP-L MSI+ tumors were aged 44 and 60 years, suggesting the underlying cause of the MSI+ phenotype was germline or somatic mutation of the mismatch repair genes rather than hMLH1 methylation". This sentence should be excluded because there is no evidence to say so in this study.

>> The suggestion is based on the evidence that MSI+ tumors from older individuals show a much higher frequency of MLH1 methylation than MSI+ tumors from younger individuals. We have added the following analysis and text to Results, p9: “In support of this, compared to other MSI+ cases (average of 0.XXX), these two
cases had significantly lower levels of \( hMLH1 \) methylation (0.XXX and 0.XXX respectively, both \( p<0.05 \))"
REVIEWER 3

Major Compulsory Revisions:

- As the authors state themselves at the end of the discussion, one of the limitations of this study, is that only one CpG site is analysed per gene with this technique, thus how valid are the interpretations of the results? As they also admit, no relation to lack of expression is validated. Shouldn’t they try to validate at least part of the results with other techniques?

>> The major goal of this study was to characterize the subgroups of colorectal cancer that are defined through the interrogation of methylation at a large number of CpG sites. With this in mind, we believe there is sufficient evidence to support the validity of the study findings, as described in the first two paragraphs of the Discussion (p13). Candidate discovery and validation were not the focus of this study.

- In the same line of thinking, it would strengthen the study if the authors would have checked whether the Weisenberger CIMP panel genes are included in the 202 set of genes analysed with the Illumina platform. Is the methylation status of these 5 genes the same in both techniques? Are they falling in the CpG loci clusters A and C?

>> We have added the following text to the Results (p11): “Unfortunately, CpG sites for only 2 \((RUNX3, IGF2)\) of the 5 genes in the CIMP\(^w\) panel were included in the Golden Gate arrays, thus preventing comparison of CIMP status by array and Methylight analysis. Nevertheless, there was good correlation between the array and Methylight methods for methylation levels of \(RUNX3\) (\(R=???,\) \(p=???)\) and \(IGF2\) (\(R=???,\) \(p=???)\).

- Also in the same line, when the authors speculate that 2 CIMP-L MSI+ tumours (44 and 60 years old) are probably mutated rather than methylated? Why did not they check the methylation status of hMLH1 in their own data? That would strengthen the assumption.

>> Please see the response to Reviewer 2 on this issue.

- Results, the clustering of the normals do show two clusters although only based in one sample. Is this normal a “real normal”? No history of previous disease or other disease besides colon? Is the adjacent mucosa from all samples real normal? Are these mucosae revised by a pathologist?

>> The normal tissues were obtained during colorectal tumor resection and were confirmed to be normal at gross examination by pathologists. Unfortunately, we have no explanation for the single “anomalous” normal sample and there was nothing distinctive about its clinical background.

- Interpretation of the results in the discussion is very limited, not to say absent sometimes. For example the authors describe the contradictions of the findings regarding mutations of KRAS but then they don’t come up with a possible reasoning. In the light of these results what is now the advice from the authors, concerning the use of a CIMP panel. Which one to use? Do we trust more the “classic” CIMP panels
or do we trust more the Illumina CIMP panels? So what is the final message of this study?

>> The final message of this study, as detailed in the Abstract conclusions, is that “Comprehensive DNA methylation profiling identified three CRC subgroups with distinctive clinicopathological and molecular features. This study suggests that both KRAS and BRAF mutations are involved with the CIMP-H pathway of CRC rather than with distinct CIMP groups”.

>> As stated in the response to Reviewer 1, we feel it is premature to comment on which panel to use until further studies are conducted (Discussion, p16): “The cost effectiveness of using arrays to characterize CIMP-H is questionable, given the strong concordance between CIMP-H from this study and CIMP\textsuperscript{w}. Further studies should clarify if the additional information provided by methylation arrays is worth the complexity and expense”

**Minor Essential Revisions:**

- Supplementary files lack detailed legends. One is not able to understand what is what, only guessing.

>> The supplementary files are annotated with data description and footnotes where necessary in the manuscript text (p27).

**Discretionary Revisions:**

- Shouldn’t the p-values and frequencies described in table 1 also be present in the body text? One should be able to read the text independently from the tables, and vice-versa.

>> Frequencies from Table 1 are mentioned in the Results and Discussion when they have been worthy of highlight. One example is in the Results, p10: “Two of the 15 MSI+ tumors were observed in the CIMP-L group and 13 in the CIMP-H group”. In many cases, p-values are not mentioned as many comparisons consider p-values from multiple tests (eg. p-values from both CIMP-H vs CIMP-M, and CIMP-H vs CIMP-L).

- It would have been interesting if the authors would have looked at more molecular features besides the ones analysed standard in literature.

>> We agree with the reviewer that knowledge of association with other molecular features would have been interesting, but unfortunately we didn’t have any further data available for this series. We reported on everything that was available.