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Comments to the Author

Title: Genome-wide methylation profiling of different stages of HBV-related HCC development in plasma cell-free DNA reveals potential biomarkers for early detection and high-risk monitoring of HCC

Author: Zhao et al.

Journal: Cancer

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General Comments:

In this study, Zhao et al. examined the methylation status of plasma cell-free DNA in healthy control, chronic HBV carriers, patients with liver cirrhosis and HCC. The authors reported certain differentially methylated regions that were associated with the different sample groups and these authors tried to correlate these observations with liver disease progression.

There are major concerns regarding the experiment design, data interpretation and writing of the manuscript.

Major comments:

1. The authors pooled plasma cell-free DNA from approx 20 individuals in each group (30ng cfDNA from each sample to make up ~500ng total input DNA) to prepare a single library per group with the pooled samples. These authors have not ruled out the possibility that the differentially methylated regions observed could be due to variations between individuals rather than the stages of the disease. It is likely that one highly methylated sample could easily significantly skew the methylation profiles deduced from MethylCap-Seq of the entire group. This raises significant concerns on the interpretation of the reported data reported and this may also explain the extremely marginal validation rate of DMRs observed.

Here, we need re-write the paper and avoid such extremely marginal validation rate. We can’t use such evidence which make our method seems very bad.

2. These authors also failed to speculate the origin of these differentially methylated cfDNAs. The authors chose 125 targets and only 33 were consistent with the observed tissue methylations. However, these authors concluded by stating that the methylation pattern of plasma DNA is consistent with those of the tissues.

Same with my prior reply. Maybe we can only said : chose 40 targets and only 33 were consistent with the observed tissue methylations

3. Many parts of the manuscript were poorly written and lacked details. For example, these authors stated “a substantial portion of the targets exhits…” without providing the exact number of samples employed.

Yes, we need to provide exact numbers.

4. Figures were of bad quality. For example, Fig 2 plotted number of reads/peaks in log scale and showed that all of the readings were very similar. This made the interpretations of the differences in the distribution of peaks very difficult.

The word “notably” cannot be used in line 39. The reads are similar for HC and other groups. Is there any inner-control for the technique of methylation-seq?

5. The hypermethylated ZNF300 was down-regulated along with the progression of HC, LC and to HCC (p<0.05). In comparison, SLC22A20 and SHISA7 did not show similar trend when coupled with gene silence pattern (Figure 4C). How about the expression and methylation status of the 3 DMGs (ZNF300, SLC22A20 and SHISA7) in the plasma samples?

It does not make sense to detect the expression of such three genes in plasma. The reviewer is stupid and he really don’t understand what we are doing.

6. Data should be provided to compare these biomarkers with the current methods of detecting HC, LC and to HCC including AFP and imaging.

Do we have AFP and image data for our samples? If we can we can do it. Otherwise, we don’t need to do it, because, we compared the diagnosis performance with biopsy.

Specific comments:

1. In M and M session for the processing of methycap-seq, there is a typo on the number of libraries generated: Five or Four?

I don’t think the reviever #1 read our manuscript carefully. Actually, we can send an argument to editor for the rejection decision because such stupid reviewer.

2. In Results section 1, how did the authors conclude a mapping rate of 50% when the height of raw reads and mapping are very close, Fig 2A? How did the authors conclude very high background levels of methylation were observed in HC samples?

Oh, yes, it seems we made a mistake. We should revise the figure 2A.

3. Figure 2B and 2C, the authors need to elaborate how they concluded methylation status accumulates with liver disease progression while NSCLC had the closest association with HCC even though it is not of liver origin. Fig 2C is of such poor quality and hence it is difficult to interpret the data properly.

I will give you another evidence to support this conclusion. Please give me a few days.

4. The authors need to explain MACS and BALM before they were used and they also needed to elaborate the difference in algorithms..

Ok. It is good suggestion, I will make some comparison and some illustration to this two method in the future version.

5. Since the authors focused on the hypermethylated regions, it was therefore unjustified to conclude in Fig 3A that there was increase in the hypermethylation in LC and HCC since there could be just as many hypomethylated regions for these samples.

Stupid question again. We do not just focus on hypermethylated region. We are interested in both hyper- and hypo-methylated regions. We also detected hypo-methylated region for LC and HCC.

6. In Fig 3B there were 240 early, 272 middle and 286 late genes based on CGI while Fig 3C could be inferred as 224 early, 231 middle and 258 late genes based on CGI only. Why was there a discrepancy?

Why 224, 231 and 258 rather than 225, 232 and 259 (as our manuscript shown, table title ?).

Here maybe therefore other aberrant DMGs occurred in some other regions such as CpG shelf, rather than CpGI and CpG shore.

7. Figure 4, the authors need to discuss the rationale for the low validation rate of plasma methylation cfDNA and tissue DNA.

Yes, we should not write such big gap between the results from tissue and serum. Maybe 20% discrete would be ok. “125 targets and only 33 were consistent with the observed tissue” it is too large to be acceptable.

8. Figure 5, the authors need to state the number of sample used for independent validation. The authors validated a total of approx 200 CpG sites and only 6 were positive. This casts doubt on the initial methodology to generate the methylation profile. Six out of 200 was a mere 3%. Is this acceptable as validation?

Here, I don’t understand what he is talking about? Why 200 CpG sites?

9. Many abbreviations were not elaborated or listed properly in the manuscript.

Yeah. We’d better to list all the abbreviations in the title page.

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Comments to the Author

The authors have to confirm the usefulness of 3 DMGs including ZNF300, SLC22A20, and SHISA7 as HCC-biomarkers using another cohort set. And they also need to reveal the function of these genes in hepatocarcinogenesis.

Ok. It’s a good idea.

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Comments to the Author

The study aimed to identify CpG sites associated with progression of HBV-related chronic liver diseases to HCC. There are several major concerns as below.

Major comments

(1)Study design is not clear. It would be better to clarify which set was used for discovery and/or model (cut-off value) training, and which set was used for validation. The predictive performance assessment with ROC should be done in independent validation set based on cut-off value(s) determined in training set without using any information from the validation set. It looks that the results presented in Figure 5 are just selection of top correlates that should be validated in another set.

这个问题很是个好问题，我们的文章确实存在这个缺陷，表面上看我们的文章内容很丰富，但是仔细分析你会发现，我们的文章是综合了生物信息学和肿瘤诊断模型两部分。但是每一部份都缺陷很大。 1）信息学是混合，很核心的结论都没法下，2）诊断模型样本量太小，没有discover, validation两个阶段。下一步我们要好好思考，到底以后采用什么样的研究模式。 应该好好研究一下Cancer research, clinical cancer research上的研究方法了。所以基于这种设计可能注定咱们这篇文章发表不到高分杂志上去。到时候是在不行，就投“Cancer Biomarkers”， Journal of Molecular Biomarkers and Diagnosis，或者plos one吧。

(2) Majority of the HCC patients are with advanced-stage diseases. This is a significant limitation in identifying DMRs associated with transition from cirrhosis to early-stage HCC as stated in Abstract as "early detection".

You are wrong, we can provide routine surveillance to HCC high risk individuals with the early HCC related methylation biomarker test so that we can give some effective suggest biomarker positive individuals to do so corresponding response to increase the death risk.

(3)Another major concern is the very small sample size. Given the inter-individual molecular heterogeneity and intra-tumoral heterogeneity in DNA methylation levels revealed in recent studies, I’m not sure if these findings are reproducible.

Yes, maybe we need to increase the sample size.

(4)More thorough tissue vs. plasma comparison should be done.

Yes, this idea is really a good idea. I think we need to publish a paper to be clear how many methylation statuses can be consistent between tissue and serum with Methylation-seq technique. I think we can publish this paper in good journals.

(5)It is not clear what each of "early", "middle", and "late" biologically/clinically indicates. It would be better to be more specific.

Ok. Let’s do it.

(6)How does the hierarchical clustering in Fig.2 inform sequential development of the disease states?

The correct conclusion is as the following:

Dendrograms are a convenient way of depicting pair-wise dissimilarity between clinical samples. In order to evaluate the correlation (similarity) or distance (dissimilarity) among HC, CHB, LC and HCC samples, we apply hierarchical clustering based dendrogram analysis to the genome-wide methylation status which is inferred by BALM method. Additionally, an independent NSCLC dataset as outgroup control was integrated into the cluster analysis to show the absolute relationship among these four groups. The hierarchical clustering result found that the methylation profile of HCC was more similar with NSCLC, and then LC, CHB, respectively which might suggest a stage-related stepwise progress process (Figure 2B)

Minor comments

1. Abstract, "ROC": "AUROC"?

I cannot find any “AUROC” in our manuscript.

(2)Methods, Statistics: What models were used for the "univariate/multivariate" analysis and how?

Both univariate and Multivariate models (adjust with age and gender) models were used to examine the association between gene CpG methylation and HCC stage.

(3)AUROC won’t be changed by modifying cut-off values.

I cannot find any “AUROC” in our manuscript.

(4)Some abbreviations are not spelled out at their first appearance. Also, too many abbreviations would cause confusion.

Ok. Let’s fix them.

(5)How does the use of MACS and BALM increase "the power of detection"?

DMGs detected by either MACS and BALM will be taken as significant DMGs, thus, significant DMGs can be detected with higher probability and with any one method. Therefore, the power of the significant detection can be higher than any one of the method.

(6)It would be better to describe methodological information rather than citing papers that need subscription.

Ok. I will do it.

(7)It would be better to describe PCR conditions.

(8)Results, first paragraph: What is "data mining"? Should be more specific.

Change “Data mining and overall features of MethylCap-seq libraries of plasma cfDNA during HCC Development” to “Characteristics of DNA methylation profile revealed by MethylCap-seq in plasma cfDNA during HCC Development”

(9)Was multiple hypothesis testing corrected in the selection of DMRs?

Yes, the multiple test corrected has been conducted in the DMR detection process. It is inbuilted by MACS and BALM.

(10)Table 1: How are the clinical demographics comparable between the sets?