Peer Review File

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Reviewer A

This paper described the function of PRKAA2 in hepatoblastoma and the usefulness of diagnostic and prognstic marker. The content of this paper is very interesting but the quality of each section was not sufficient to be accepted. The authors should reconsider to describe these data for publication. If possible, the author might divide this paper into two papers: basic of the function of PRKAA2 in hepatoblastoma and clinical application of this marker.

Reply:

Thank you very much for your suggestions. Following your advice has improved the quality of my article.

In this article, by studying the mechanism of its high expression and its cancer-promoting effect, the regulation of PRKAA2 on ferroptosis may be an important molecular mechanism of hepatoblastoma (HB), which provides a basis for PRKAA2 as a disease marker. The function of PRKAA2 in HB has not been reported. Functional experiments can provide stronger support for the clinical application of PRKAA2, so after careful consideration, we still hope to integrate the two parts in this manuscript.

To improving the structure of the manuscript, we re-distributed the content, kept the most core experimental results in the main body, and put other results in the supplementary file, hoping to make readers more easily understand our research.

On the whole, this paper firstly used public data to screen possible HB biomarkers through bioinformatics methods, then used paired samples collected by our team based on the Shanghai children's medical center platform to verify the expression of screened genes and conduct clinical value research, and finally verified whether the key gene affect HB cell proliferation and tumor growth through *in vivo* and *in vitro* experiments. And explored the mechanism through which it exerts biological effects.

Comment 1:

Line 220: “Data from 103 patients with HB and 19 controls from the GSE131329 and GSE75271 datasets”

Did you confirm these datasets from the tumors which were not treated? This is most critical point for this paper. The authors should clarify the source of these dataset. Moreover, what were the 19 controls? Are these derived from healthy children?

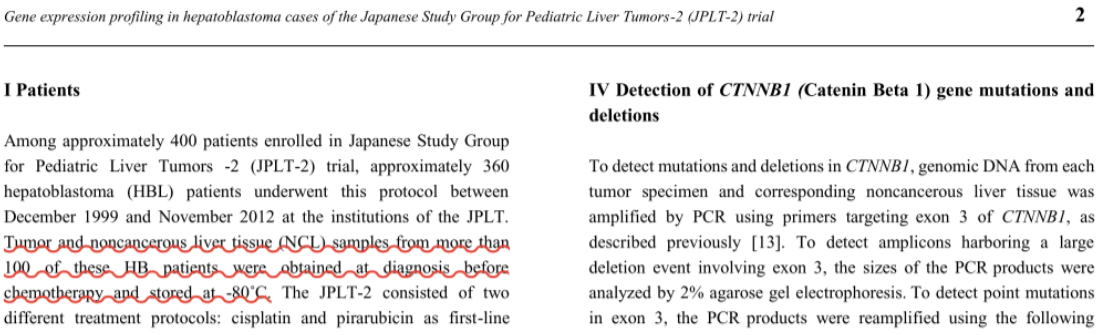
Reply 1:

Thank you for your suggestion and we are sorry that we did not elaborate on this issue before, we have added it in the manuscript.

Both datasets are from the GEO database. GSE131329 is derived from the article "Gene expression profiling in hepatoblastoma cases of the Japanese Study Group for Pediatric Liver Tumors 2 (JPLT-2) trial", and GSE75285 is derived from the article "Genomic analysis of hepatoblastoma identifies distinct molecular and prognostic subgroups".

In both articles, the authors made it clear that all samples were obtained before the patients were treated, and the control samples were derived from corresponding noncancerous liver tissues. We took a screenshot of this information, as shown below.

GSE131329



Gene expression profiling in hepatoblastoma cases of the Japanese Study Group for Pediatric Liver Tumors-2 (JPLT-2) trial 2

I Patients

Among approximately 400 patients enrolled in Japanese Study Group for Pediatric Liver Tumors -2 (JPLT-2) trial, approximately 360 hepatoblastoma (HBL) patients underwent this protocol between December 1999 and November 2012 at the institutions of the JPLT. Tumor and noncancerous liver tissue (NCL) samples from more than 100 of these HB patients were obtained at diagnosis before chemotherapy and stored at -80˚C. The JPLT-2 consisted of two different treatment protocols: cisplatin and pirarubicin as first-line

IV Detection of *CTNNB1* (Catenin Beta 1) gene mutations and deletions

To detect mutations and deletions in *CTNNB1*, genomic DNA from each tumor specimen and corresponding noncancerous liver tissue was amplified by PCR using primers targeting exon 3 of *CTNNB1*, as described previously [13]. To detect amplicons harboring a large deletion event involving exon 3, the sizes of the PCR products were analyzed by 2% agarose gel electrophoresis. To detect point mutations in exon 3, the PCR products were reamplified using the following

GSE75285

prognostic groups and biomarkers for other embryonal tumors[3] as well as adult hepatocellular malignancies.[4-6] For example, survival-predictive and metastasis-predictive biomarkers based on both gene and microRNA (miRNA) expression profiles have been reported for hepatocellular carcinoma (HCC),[7] the most common liver tumor in adults.[4] Interestingly, a "stem-cell" gene-expression signature, involving several oncofetal, stem-cell markers, and pluripotent stem-cell expression profiles, has been identified in a particularly aggressive type of HCC.[4,8-10]

Results from recent international HB clinical studies suggest that underlying biological differences may be

that are differentially expressed in HB relative to HCC and normal liver.[18] Multiple studies have speculated about the biological and prognostic importance of specific genes and signaling pathways, but these are limited by the availability of HB tumor specimens, most of which have been postchemotherapy samples with incomplete clinical annotation.[19]

The goal of our study was to molecularly characterize a large cohort of pretreatment, clinically and histopathologically annotated HBs of sufficient size to identify significantly predictive diagnostic and prognostic biomarkers in this disease. Conclusions from previous

We are very sorry for the misunderstanding caused by our original use of "normal" to describe the controls in the manuscript, and we have replaced all "normal" with "noncancerous". Hope to make it easy for readers to understand.

Changes in the text:

we have modified our text (see Page 12-13, line 237-241; Page 6, line 93,94,97; Page 7, line 115, 121; Page 8, line 128; Page 14, line 274, 277; Page 15, line 284, 303).

Comment 2:

Line 223 Figure 1 is not described with sufficient figure legends. The authors should remake this figure with sufficient figure legends.

Reply 2:

Thank you for reminding us. We have supplemented the legend of Figure 1.

Changes in the text:

we have modified our text as advised (see Page 34, line 593-600).

Comment 3:

Lines 224-5: The results of DE-FRGs were not well described in this section and Fig.1.

Reply 3:

Thank you for reminding us that we were less descriptive of the results in this section. Due to the limitation of figure size, we only show the critical information, and more detailed content was originally shown in the supplementary tables.

According to your suggestion, we have modified this. We put the two supplementary tables in the main text. The figures and tables were too far apart for easy reading, so we decided to delete Figures 1C and 1D and present the results of the functional analysis directly in tabular detail, hoping that readers can understand our research content more clearly.

Changes in the text:

we have modified our text and figures (see Page 13, line 243-250, 252-254, 256-257; Page 28-29, line 574-577; Page 34, line 593-600).

Comment 4:

Lines 231-236: The data of two algorisms: Lasso regression and SVM-RFE algorithm were not well described. The authors should explain these result in detail in this section and Fig.2. Where is 11 variables?

Reply 4:

Thank you for your suggestion. We have added a description of the 11 genes screened by the LASSO regression algorithm and the 4 genes screened by the SVM-REF algorithm to the revised manuscript.

I am sorry that we did not show the 11 variables clearly in the figure. The position between the two dashed lines in Supplementary Figure 1A indicates the range in which the cross-validation error is minimized, and the genes are selected within this range. In Supplementary Figure 1B, 11 genes selected within this range are shown (11 different colored lines).

We have added a simple description of the rules of the two algorithms and cite two literatures in the text, hoping to help interested readers understand these two algorithms.

Changes in the text:

we have modified our text and figures (see Page 14, line 260-270; Page 34, line 601-604; Page 38, line 654-658).

Comment 5:

Lines 256-7, the PRKAA2 is related to neutrophils, macrophages M1, macrophages M2, mast cells activated, and mast cell resting. These phenomena could not be found.

Reply 5:

We are very sorry that our description of the result may have caused your misunderstanding. The results of this part are shown in Supplementary Figure 2B. CIBERSORT was used to calculate the proportion of 22 types of infiltrating immune cells in tissues. Combining support vector regression with prior knowledge of expression profiles from purified leukocyte subsets, CIBERSORT can accurately estimate the immune composition of a tumor biopsy. The middle row shows the correlation between PRKAA2 expression and 22 immune cells. Among 22 immune cells, neutrophils, macrophages M1, macrophages M2, mast cells activated, and mast cells resting were significantly correlated with PRKAA2 expression.

We have replaced "xx is related to xx" with "xx expression is correlated with xx". The hope is to convey more accurate information to readers. Considering that there are too many graphs in this paper, we decided to put these figures in the supplementary file. The current analysis of immune infiltration has not been verified experimentally. We then considered performing immunological experiments to address this finding, which might yield other interesting findings.

Changes in the text:

we have modified our text and figures (see Page 8, line 128-132; Page 15, line 285-298; Page 22, line 438-443).

Comment 6:

Lines 267-9: How did the authors divide the low and high expression of this PRKAA2? The exact data of Δ Ct should be described.

Reply 6:

Thank you for the detailed review, this is indeed the calculation method that is necessary to make clear to readers. We are sorry we made a writing error. "ΔCt" should be "ΔΔCt". We analyzed the results of qRT-PCR to obtain ΔΔCt and find the middle value. Tumor tissues with higher ΔΔCt than the middle value were PRKAA2 highly expressed, and tumor tissues with lower ΔΔCt than the middle value were PRKAA2 low-expressed, and the middle value was 2.50748457256287. The specific data of ΔΔCt we added in the section of supplementary appendix. For the calculation principle of ΔΔCt, please refer to this article: Statistical analysis

of real-time PCR data. (This article is available from: http://www.biomedcentral.com/1471-2105/7/85)

Changes in the text:

we have modified our text (see Page 16, line 310-311; Page 44-45, line 716-718).

Comment 7:

Line 286. What is siPRKAA2-2? If the several siRNAs were used, the authors described these and the data of all si-RNA.

Reply 7:

We are sorry that we didn't give a clear description here, which caused your misunderstanding. We have added descriptions in this position. We used two PRKAA2 siRNAs, and the specific sequence information is shown in the supplementary material. Experiments verified that the interference efficiency of siPRKAA2-2 was better, so we chose to design PRKAA2-knockdown lentiviral plasmid based on siPRKAA2-2 for subsequent experiments.

Changes in the text:

we have modified our text (see Page 17, line 330-331).

Comment 8:

In Tables 1, 2, and 3,

Why were the AFP levels divided into two groups with 1200 ng/ml? In the patients with hepatoblastoma, low AFP levels is usually considered as a poor prognostic indicator. However, in this cohort, all 5 cases whose AFP levels were less than 1,200 showed low PKAA2. These patients were very interesting so that the authors should describe these patients in detail: age at diagnosis, histology, PRETEXT and annotation factors.

Reply 8:

Currently, in the clinical diagnostic criteria of liver cancer in China, the diagnostic cut-off value of AFP should be ≥400 ng/ml. However, in clinical practice, this criterion does not apply to liver tumors in children. We first selected 400 ng/ml for analysis and found that a valid analysis could not be performed in our samples. This is because the AFP level of HB children is often very high, with very few children below 400 ng/ml and even some children

reaching more than 12000 ng/ml. After consulting clinicians, it was learned that often more than 1200 ng/ml indicated liver malignant tumors and some other reproductive system tumors, and benign liver lesions often did not exceed 1000 ng/ml. So we chose 1200 ng/ml for our analysis.

Additional detail about the five patients is tabulated as supplementary information. It has been reported that HB patients with an AFP <100 ng/mL had poor prognoses (cite an article for your reference: PMID: 33883936). However, the AFP values of these five patients were all higher than 100 ng/mL, and these 5 patients had no poor prognostic indicator such as PRETEXT stage IV, metastasis and multiple foci of the primary tumor. So there is no evidence that these patients have poor prognosis. These patients were very interesting, we hypothesized that low PRKAA2 expression as well as low AFP level (1200ng/mL > AFP > 100 ng/mL) might be a good prognostic factor. However, this needs to be verified by further expanding the sample size.

Thank you for providing a very good idea for our follow-up research. We hope to have another opportunity to discuss this issue with you.

Changes in the text:

we have modified our text (see Page 21, line 423-431; Page 44, line 714).

Comment 9:

In these tables, the authors have to add the data of annotation factors in PRETEXT classification.

Reply 9:

Thank you for reminding us that we only considered the extent of tumor within the liver when we conducted the correlation analysis. We have added annotation factors including vascular involvement, tumor rupture, multifocality, extrahepatic spread and caudate involvement in the tables. The correlation analysis of PRKAA2 expression and lymph node metastases could not be performed because all 30 patients did not have lymph node metastasis, therefore in the tables we did not add lymph node metastasis. Through analyzing the data of annotation factors, it was found that multifocality is correlated with PRKAA2 protein expression levels.

Changes in the text:

we have modified our text (see Page 16, line 306-308, 316; Page 29, line 578; Page 30, line 582; Page 31, line 588). We uploaded these data separately in the EXCEL file.

Comment 10:

In all figures,

The graphs and photographs are too small and too many in each figure to be understood. And the figure legends are insufficient to explain these figures. The authors should remake these figures to be understood easily by readers.

Reply 10:

Thank you for your suggestion. We should take the readers' feelings into full consideration. We reconsidered and reworked all the figures, keeping the core graphs and moving some of the content to the supplementary figures. All the figure legends have also been rewritten to make it easier for readers to understand our content.

Changes in the text:

we have modified our text and figures (see Page 33-42, line 592-693).

Comment 11:

There are several grammatical errors.

Line 45-6 a potential diagnostic and prognostic markers.

Reply 11:

We must apologize for our carelessness. Thank you very much for finding our grammatical errors. We have asked native English speaking colleagues to revise the full text.

Changes in the text:

we have modified our text and figures (see Page 3, line 46).

Reviewer B

1. Line 143: Pls be specific on the IRB's name.

Reply:

Thank you for reminding us. This study was approved by the ethics committee of Shanghai Children' s Medical Centre.

Changes in the text:

We have modified our text (see Page 9, line 145-146).

2. Line 145:

Please confirm if the study was conducted in accordance with the Declaration of Helsinki (as revised in 2013)., and add the statement here. Available at: https://www.wma.net/wp-content/uploads/2016/11/DoH-Oct2013-JAMA.pdf

Reply:

Thank you for reminding us.

This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

In the previous version we only added this statement in the footnote section, now we have added it here as well.

Changes in the text:

We have modified our text (see Page 9, line 147).

3. Line 478: You provided two versions of contradictory statements on consents. Pls check and revise.

Reply: We are very sorry for this mistake. In our study, all patients or their guardians provided their verbal and written consent.

Changes in the text:

We have modified our text (see Page 24, line 483).

4. Line 492: Ref 4 and 38 are duplicate. Pls revise.

Reply : We are very sorry for this mistake. We removed the duplicate reference.

Changes in the text:

We have modified our text (see Page 30).