Rebuttal letter

Dear Prof. Vladimir Uversky,

Thank you so much for your kind considerations and suggestions, which are very important to improve the quality of our manuscript (The novel role of LDHA/LDHB in the prognostic value and tumor-immune infiltration in clear cell renal cell carcinoma, #83877).

The reviewers' comments helped us so much in presenting our findings. We followed the reviewers' comments carefully, and performed necessary improvements in the revised manuscript. Attached are our point-by-point responses to the editor's and reviewers' comments. We truly appreciate your consideration and would be very grateful for the opportunity to publish in your esteemed journal.

Cao Yehua help to revise the manuscript and rephrase the figures/tables, and all of us agree to accept her as one of the authors. We hope to meet with your approval.

Thank you for your helpful suggestions again, and we look forward to hearing from you soon.

Yours sincerely,

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Response to Editor

Editor's comment:

As you can see, all four reviewers provided very supportive comments. However, they also indicated that some amendments are needed. Therefore, please address the concerns of all reviewers and revise your manuscript accordingly.

[# PeerJ Staff Note: Please ensure that all review and editorial comments are addressed in a response letter and any edits or clarifications mentioned in the letter are also inserted into the revised manuscript where appropriate. #]

Point-to-point response: Thank you for your helpful suggestion. This is a good opportunity for us to improve the quality of our manuscript. We ensure that all review and editorial comments are addressed in a response letter and any edits or clarifications mentioned in the letter are also inserted into the revised manuscript where appropriate. Thank you again.

Response to Reviewers

1. The comment of Reviewer 1:

1.1 Basic reporting

The language is clear and well written. The authors have provided enough references to support their statements made in the introduction as well as discussions. There is right amount of background information available. All necessary figures/tables and raw data are shared clearly.

Point-to-point response: Thank you for your helpful suggestion.

1.2 Experimental design

The research is meaningful and needed, it has been well studied in other types of cancer, however there has been no clear correlation btw LDHB and tumor immune response. But this research would serve as a good starting point to further explore this relationship. The methods are clearly written and have detailed information for future studies.

Point-to-point response: Thank you for your helpful suggestion.

1.3 Validity of the findings

The rationale is clearly stated and has been validated by providing substantial data and most of the results have been statistically valid. The authors have used normal tissues as controls. The conclusions are well stated and have also mentioned the drawback/limitations of the manuscript.

Point-to-point response: Thank you for your helpful suggestion.

1.4 The reviewer has attached an annotated manuscript to this review.

Point-to-point response: Thank you for your helpful suggestion. We clarified these issues and revised these data (Line 206, 208 in former manuscript; Line 207, 209 in revised manuscript) in the revised manuscript according to your suggestions. Just one question, there is no space between 'LDHB' and 'were' of the Abstract paragraph before the title page. We have tried many times, but failed. There might be the error of the web page. Could the editor help us? Thank you again.

2. The comment of Reviewer 2:

2.1 Basic reporting

The research manuscript "The novel role of LDHA/LDHB in the prognostic value and tumor-immune infiltration in clear cell renal cell carcinoma" by Chen et al investigates prognostic significance of LDHA/LDHB, the critical components of glycolytic enzyme LDH in clear cell renal cell carcinoma. Authors have investigated expression of LDHA and LDHB in cancerous and normal tissues by various experimental techniques including, expression profiling by (GEPIA2), western blotting and immunohistochemistry. Research findings from the study suggests a significantly elevated mRNA expression of LDHA in cancerous samples compared to the normal tissues. Expression of LDHB and LDHD mRNA was found to be low, whereas LDHC expression level didn't had any change in cancerous samples compared with normal tissues. It's clinically interesting to observe direct correlation between tumor size, grades and LDHA expression.

2.1.1. Line 28-29 is complex and needs fragmentation for improved understanding and clarity in the flow. Manuscript at several places need improvement in English. Kindly, recheck the manuscript for grammar and flow.

Point-to-point response: Thank you for your helpful suggestion. We rectified the defects accordingly in the revised manuscript, i.e., Line 28-29 "Lactate dehydrogenase (LDH), a crucial glycolytic enzyme, mediates the metabolic plasticity of cancer cells, whereas its clinical significance in renal cell carcinoma (RCC) is poorly understood" was revised to "Lactate dehydrogenase (LDH) is a crucial glycolytic enzyme which mediates the metabolic plasticity of cancer cells, however its clinical significance in renal cell carcinoma (RCC) is poorly understood (line 29-30 in revised manuscript)". We also recheck the manuscript for grammar and flow in the revised manuscript. Thank you again.

2.1.2. The study hasn't explored the mechanism behind the down-regulated expression of LDHB. It would add value to explain the possible cause in the discussion section.

Point-to-point response: We appreciate the reviewer's feedback and agree that exploring the mechanism behind the down-regulated expression of LDHB would add value to our study. While we did not specifically investigate the underlying cause of LDHB downregulation in our study, previous studies have suggested that epigenetic modifications such as DNA methylation and histone deacetylation, as well as dysregulated microRNA expression, may be involved in regulating LDHB expression. We have added a brief discussion on these potential mechanisms in the revised manuscript. However, further research is needed to fully understand the regulatory mechanisms of LDHB expression in ccRCC. Please let us know if you have any additional concerns or suggestions. Thank you again for your helpful feedback.

The following paragraph is attached in the discussion:

LDHB downregulation has been observed in various types of cancer, and it is important to understand the underlying mechanisms behind this phenomenon. Epigenetic modifications, such as DNA methylation and histone deacetylation, have

been shown to play a role in regulating LDHB expression (de Mello et al. 2017). DNA methylation is an epigenetic modification that involves the addition of a methyl group to the cytosine residue of CpG dinucleotides. Hypermethylation of the LDHB promoter region has been reported in several types of cancer, including gastric cancer, hepatocellular carcinoma, and pancreatic cancer (Maekawa et al. 2003). In these cases, hypermethylation of the promoter region results in the silencing of LDHB expression. Histone deacetylation is another epigenetic modification that can lead to gene silencing. The histone deacetylase inhibitor trichostatin A has been shown to upregulate LDHB expression in breast cancer cells (Rodrigues et al. 2015). Dysregulated microRNA expression has also been implicated in the downregulation of LDHB expression (Frank et al. 2021). MicroRNAs are small RNA molecules that negatively regulate gene expression by binding to the 3' untranslated region (UTR) of target mRNAs and causing their degradation or translational repression (This needs citation). Several studies have identified specific microRNAs that target LDHB. For example, miR-375 has been shown to downregulate LDHB expression in breast cancer cells (Frank et al. 2021). In summary, the downregulation of LDHB in cancer might be due to epigenetic modifications such as DNA methylation and histone deacetylation, as well as dysregulated microRNA expression. Understanding the mechanisms behind LDHB downregulation may help to identify potential therapeutic targets for cancer. Are there public datasets of Renal Cell Carcinoma where authors could look at miRNA data to see if miR-375 is increased?

2.2 **Experimental design**

The study design is meaningful and satisfactory for the most parts. The research findings are well supported by figures and tables. The lack of data regarding the functional mechanism involved needs explanation in the text.

Point-to-point response: Thank you for your helpful suggestion. Yes, we agree that it is important to clarify the underlying mechanism. We have added a brief discussion on these potential mechanisms in the revised manuscript (See 2.1.2). And this also points out the direction for our further exploration. Thank you so much for your feedback again.

2.3 **Validity of the findings**

The study is of high clinical relevance as it also shows LDHB as an independent predictor of overall survival in patients with ccRCC. However, as already mentioned by the authors the study lacks the mechanism behind the deregulated expression and future studies are warranted to explore the mechanism further. The study can be accepted for the publication based on clinical significance. However, a few minor points need to be explained.

Point-to-point response: Thank you for your helpful suggestion. We appreciate the positive feedback from the reviewer regarding the clinical relevance of our study, and we agree that further studies are warranted to explore the mechanism behind the deregulated expression of LDHB. We have added a brief discussion on potential mechanisms, as suggested by the reviewer, in the revised manuscript (See 2.1.2). Thank you again.

2.4 **Additional comments**

Can be accepted after a minor revision.

Point-to-point response: Thank you for your helpful suggestion.

3. The comment of Reviewer & Prof. Shruti Singh Kakan:

3.1 Basic reporting

no comment

Point-to-point response: Thank you for your helpful suggestion.

3.2 **Experimental design**

Methods related suggestions - Please update the 'Statistical Analysis' section addressing the major comments 4 and 5, included below.

Point-to-point response: Thank you for your helpful suggestion. We clarified these issues (revised these data) in the revised manuscript according to your suggestions. Thank you again.

3.3 Validity of the findings

Please address all comments included in the additional comments section (major comment 8) below.

Point-to-point response: Thank you for your helpful suggestion. We clarified these issues (revised these data) in the revised manuscript according to your suggestions. Thank you again.

3.4 Additional comments

In this manuscript, authors have investigated the expression profiles of metabolic enzymes LDHA and LDHB in clear cell renal cell carcinoma (ccRCC) compared to healthy renal tissue. Authors found, using IHC, WB as well as public databases (TCGA) that protein expression of LDHA was positively associated with poor survival and that in case of LDHB, protein expression was negatively associated with overall survival in ccRCC tissue. Additionally, authors also observed that LDHB was negatively associated with tumor aggressiveness and could predict survival in ccRCC patients independently of other factors. This is the most important finding of the paper.

Using additional data mining, authors report a strong correlation between LDHA and CD4+ T cells as well as LDHB and CD4+ T cells in addition to other immune cell types.

Overall, this is a fairly well written manuscript which is within the scope of the journal and fairly scientifically sound as it employs multiple experimental methods (both WB and IHC in addition to public datasets) to confirm its major finding. I have the following comments which can help improve the manuscript further.

Point-to-point response: Thank you for your helpful suggestion. This is a good opportunity for us to improve the quality of our manuscript. Thank you again.

3.4.1 Major comments

1). Prior to conducting WB or IHC, what methods (or cancer markers) were used to confirm the tissues imaged were indeed cancerous? Similarly, what markers were used to confirm that adjacent kidney cells were non-cancerous

Point-to-point response: Thank you for your feedback. When we archived the tissues, we could find that the tissue morphology is different between cancerous and non-cancerous tissues. There are distinctly characteristics between cancerous and non-cancerous tissues, i.e., the profile of cancerous tissue is fragile and colorful, however

non-cancerous tissues are toughness and red. Before conducting WB or IHC, we also embedded the tissues in the paraffin and performed conventional hematoxylin-eosin (HE) staining, which could confirm their nature. Thank you again.

Please include this data in the supplemental, and text in the main manuscript results section explaining the morphological characteristics of the tissue types as assessed by H&E.

2). Though the association with metastatic status is not statistically-significant, it may yet be biologically relevant and the authors should note this either in results or discussion.

Point-to-point response: Yes, I totally agree with you. The metastatic status is a critical characteristic of cancer progression, and is usually a poor prognostic marker of cancers. We found that LDHB was negatively correlated with metastasis of ccRCC (P=0.001), and LDHA was potentially positively correlated with metastasis of ccRCC (P=0.059), which has been put in the result. Thank you again.

3). How do authors reconcile the conflicting results of the correlation of CD8+ T cells and LDHA expression between TISIDB and TIMER analysis?

Point-to-point response: Thank you for your helpful suggestion. In the manuscript, LDHA was reversely associated with CD8+ T cells (rho = -0.243, $P < 0.001$) using TIMER analysis, whereas LDHA was positively associated with central memory $CD8^+$ T cell (Tcm_CD8, rho=0.301, $P<0.001$) using TISIDB analysis. TIMER evaluated the abundance of 8 tumor-infiltrating immune cells (TIIC) subsets in the ccRCC cohort, and TISIDB elucidated the abundance of 28 tumor-infiltrating lymphocytes (TILs) in ccRCC. And Tcm_CD8 cells is just a part of CD8+ T cells. We hope to clarify this ambiguity. Please let us know if you have any additional concerns or suggestions. Thank you again for your helpful feedback.

Thank you for the clarification. Please also clarify in the text, wherever possible in parentheses, that Tcm_CD8 are a subset/(memory cell) subtype of CD8+ T cell.

4). In some cases (for e.g., the WB samples), patients have paired data; did authors

use appropriate paired tests to assess statistical differences? It is not clear.

Point-to-point response: Thank you for your helpful suggestion. According to your suggestion, "Student's t-test was performed for WB analysis to evaluate LDHA and LDHB expression" was revised to "Paired student's t-test was performed for WB analysis to evaluate LDHA and LDHB expression". Thank you.

5). In Results, lines 241 onwards, would it be prudent to use a multiple testing correction here?

Clearly, CD4+T cells correlate highly with LDHB, more than any other immune cell type and a multiple comparison p-value correction would capture this. I recommend authors to verify this analysis by a statistician.

Point-to-point response: Thank you for your helpful suggestion. We utilized multiple testing methods, including K-M survival analysis, univariate Cox regression analysis and multivariate Cox regression analysis (Lines 241 onwards). Each statistical testing has its own meaning, especially multivariate Cox regression analysis. Multivariate Cox regression analysis could identify the real influencing factor(s) from many confounding factors. Here we recognized pathological stage and LDHB as independent prognostic indicators for OS in ccRCC patients.

Yes, CD4+T cells correlate highly with LDHB using TIMER analysis. Multiple algorithms, including TIMER, OBERSORT, XCELL, and EPIC, were applied for immune infiltration estimations, which all confirmed their tight correlation. We also verify this analysis by a statistician. Thank you again.

6). Another limitation is the lack of independent validation at protein level either through IHC or IF or flow cytometry of LDHA/LDHB levels in tissue with immune cell subtypes. Authors should address this in the Discussion.

Point-to-point response: Thank you for your helpful suggestion. "The third limitation is the lack of independent validation at protein level either through IHC or flow cytometry of LDHA/LDHB levels in tissue with immune cell subtypes". This sentence has been inserted in the Discussion in the revised manuscript. Thank you.

7). Why did the authors use DAB and not ECL for WB. ECL is much more sensitive than DAB which is also very toxic.

Please also mention In Methods how the developed Western Blots were imaged?

Can WB data be quantified using ImageJ? (The comment in the annotated manuscript)

Did authors explore other methods of testing tumor tissue for LDHA/LDHB which have higher sensitivity than IHC or WB? This could be discussed in discussions.

Point-to-point response: Thank you for your reminding. We checked our record and found ECL instead of DAB was used for WB detection. We have rectified this error in the revised manuscript. Thank you so much for your reminding.

We captured the images of WB bands using Gel Image System FluorChem M (ProteinSimple, USA), and "captured by Gel Image System FluorChem M (ProteinSimple, USA)" was added in the revised manuscript.

Yes, we utilized ImageJ to quantify WB bands, which was also cited in the research paper (PMID: 33925918). Thank you.

Thank you for your helpful suggestion. Actually, we did not explore other methods (such as Q-PCR) of testing tumor tissue for LDHA/LDHB using our archived tissues, but large samples data from GEPIA2 and UALCAN have already confirmed LDHA/LDHB expression in tumor tissue. And we discussed this in the discussion.

8). In Figure 3, I observe a variation in the tissue types imaged. Particular, the low expression group from the LDHA, it appears only fat cells have been imaged. Please also comment on the morphological changes ocurring due to the tumor itself. But for IHC comparisons, please try to image similar renal tissue as much as possible.

In the Figure 3 itself, please label tissue type in each of the three IHC panels.

Point-to-point response: Thank you for your helpful suggestion. Yes, we agree with you. ccRCC is characterized as amount of lipid droplets (LDs), i. e., lipid and glycogen, in the cytoplasm. During the HE or IHC staining, the LDs tend to be resolved and form a vacuolar appearance, especially for the low grade ccRCC

specimens. Therefore, there is more fat-like cells [actually low grade (G1) ccRCC cells] in the low expression group from the LDHA.

In the Figure 3, we labeled tissue type in each of the three IHC panels in the revised figure legends. To be specific, normal kidney tissue, ccRCC (G1), and ccRCC (G2) were listed in the upper lane (LDHA), whereas normal kidney tissue, ccRCC (G3), and ccRCC (G2) were listed in the lower lane (LDHB). Thank you.

Thank you for including information regarding lipid droplets in ccRCC tissue. This is helpful to the reader andd I recommend that authors add this information in the text.

9). In the supplemental dataset file 1, please mark the lane numbers, the marker being looked at and the molecular weights.

Why was the gel membrane cut?

Point-to-point response: Thank you for your helpful suggestion. In the revised supplemental dataset file 1, we have marked the lane numbers, the marker being looked at and the molecular weights, alongside with the original gel membrane.

Please let us know if you have any additional concerns or suggestions. If possible, we could repeat the WB experiment using the same tissue lysates. Thank you again.

In the lab, we usually cut the membrane to a small piece and keep the appropriate molecular weight gel/membrane, then the membrane is incubated with secondary antibody. This method could save the antibodies and reagents, but sometimes also misses much useful information. Thank you again.

Thank you for including this information.

WB MW usually go from higher MW to lower MW, in decreasing order. But in the supplemental data file, the MW markers in the supplemental are reversed (going from 40 Da to 35 kDa. Please explain.

Minor comments

1). The abstract has sufficient information to stand on its own.

On line 29, ‘however’ would be appropriate rather that ‘whereas'

Point-to-point response: Thank you for your helpful suggestion. We have changed ‘whereas' to ‘however’ in the revised manuscript. Thank you again.

2). In Introduction

-line 86, Please include which study was the analysis done in?

-line 92 says 'tumor immune?' This sentence feels incomplete

-Lines 96-99, Please mention if this was in the same set of patients or a separate validation cohort of 150 patients.

Point-to-point response: Thank you for your helpful suggestion. We revised the sentences according to your suggestions. Thank you again.

-line 86, we have rephrased 'Our previous proteomic analysis identified numerous dysregulated proteins, such as hydroxy acyl-CoA dehydrogenase alpha subunit (HADHA), LDHA, and LDHB, which might be implicated in RCC pathogenesis (Zhao et al. 2015)' to 'Using quantitative proteomics analysis, our previous study identified numerous dysregulated proteins, such as hydroxy acyl-CoA dehydrogenase alpha subunit (HADHA), LDHA, and LDHB, which might be implicated in RCC pathogenesis (Zhao et al. 2015)' in the revised manuscript.

--line 92, we rectified 'tumor immune' to 'tumor immune infiltration' in the revised manuscript.

-Lines 96-99, Yes, we confirmed this was in the same set of patients of 150 patients. And we rephrased '…, assessed their prognostic role in the 150 ccRCC patients, …' to '…, assessed their prognostic role in the same 150 ccRCC patients, …' in the revised manuscript.

Validation cohort is typically a separate group as this makes for a stronger validation.

3). In Methods, lines 145-146, what was configuration of the microscope, year, model, etc.

Point-to-point response: Thank you for your helpful suggestion. The model (configuration) and year of the microscope were Olympus BX53, 2013. We revised the sentences according to your suggestions. Thank you again.

4). In Methods, Please specify that the GEPIA2 database uses TCGA datasets. Please also include appropriate citations and links to the publicly available datasets throughout the methods.

Point-to-point response: Thank you for your helpful suggestion. Yes, we specify that the GEPIA2 database uses TCGA datasets. And we also include appropriate citations

and links to the publicly available datasets throughout the methods. In the method, 'GEPIA2 was also used to validate the relationship ….' was revised to 'GEPIA2 (http://gepia2.cancer-pku.cn/) from TCGA was also used to validate the relationship …'. Thank you again.

5). In Results lines 207-217, please include statistical test next to P values. Authors can also include expression level averages with SD in the same parentheses.

Point-to-point response: Thank you for your helpful suggestion. We revised the sentences according to your suggestions, for example, '(P<0.001)' was changed to '(P<0.001, Pearson chi-square test, or Fisher exact test)'. For concision and succinct, we did not include expression level averages with SD in the same parentheses. I can provide this detailed data if you have any additional concerns. Thank you again.

6). In Discussions, line 380 Which limited sample size are the authors referring to? Please be specific.

Point-to-point response: Thank you for your helpful suggestion. We revised the sentences according to your suggestions, i.e., changed 'the limited sample size' to 'the limited sample size of the IHC analysis'. Thank you again.

7). In Figure 2B and 2C, Please include all points in bar plot & box and whisker's plot similar to figure 2A

Point-to-point response: Thank you for your helpful suggestion. We revised the Figure 2B and 2C according to your suggestions. Thank you again.

8). In Figure 5, please explain the difference between the labels Tem_CD8 and Tcm_CD8

Point-to-point response: Thank you for your helpful suggestion. Tem_CD8 is the abbreviation of 'effector memory CD8+ T cell' and Tcm_CD8 denotes 'central memory $CD8^+$ T cell'. We have put these data in the manuscript and the legend of Figure 5 in the revised manuscript. Thank you again.

9). Did the authors consider using Figure 6 data for pathway analysis and for hypothesis generation on the relationship between LDHB, immune cell subtypes and immune inhibitory molecules?

Point-to-point response: Thank you for your helpful suggestion. This is a good idea, and we tried to hypothesize the pathway or relationship between LDHB, immune cell

subtypes and immune inhibitory molecules using online databases. STRING (www.string-db.org) and DAVID (https://david.ncifcrf.gov/) display that LDHB does not interact with other immune inhibitory molecules (Figure a, b), which indicates the novel role of LDHB in tumor immune infiltration. And we hope to clarify their relationship for the further research. Thank you again.

I recommend authors explore the LDHA & LDHB pathways in Reactome & wiki pathways and do a broader gene ontology (cellular processes, biological functions) assessment. Looking at only immune inhibitory molecules could be limiting as LDHAs connection to immune processes may be indirect or may be cellular trafficking related.

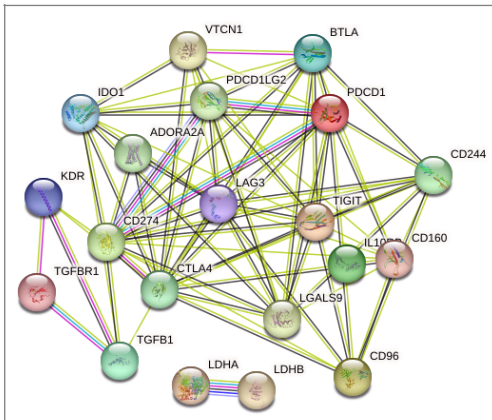


Figure a: LDHB and immune inhibitory molecules

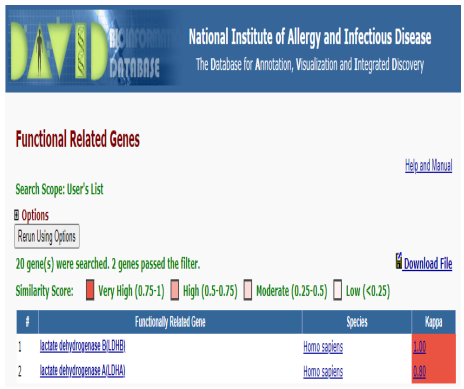


Figure b: LDHB and immune inhibitory molecules (DAVID).

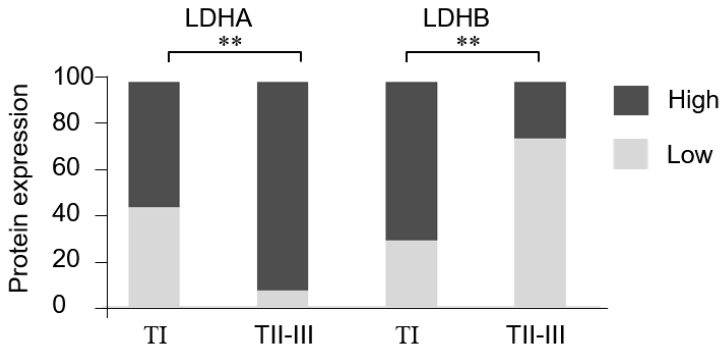
10. In Table 1, Please include what the staining values mean, and how they were obtained in the table footer.

Do the LDHA and LDHB staining values correlate with ccRCC stage. If so, can authors include a figure showing that.

Point-to-point response: Thank you for your helpful suggestion. The staining values mean LDHA or LDHB immunoexpression, which was scored by evaluating the cytoplasmic staining intensity (0~3) and frequency (0~4) as previously described (PMID: 33027752). According to their expression, they were classified into two groups: low group (cancer scores <5 for LDHA, <6 for LDHB) and high group (scores ≥5 for LDHA, ≥6 for LDHB). Please include this in the methods.

Yes, the LDHA and LDHB staining values correlate with ccRCC stage (Table 1). And

we put the corresponding figure showing the relationship between LDHA/LDHB staining values and ccRCC stage in the supplementary figure 2a.



11. note: The reviewer has attached an annotated manuscript to this review.

Point-to-point response: Thank you for your helpful suggestion. We clarified these issues (revised these data) in the revised manuscript according to your suggestions. The following responses is attached here. Thank you again.

1)Can WB data be quantified using ImageJ? (The comment in the annotated manuscript)

Point-to-point response: Thank you for your helpful suggestion. Yes, we utilized ImageJ to quantify WB bands, which could be found in the research paper (PMID: 33925918). Thank you.

2) Please include the statistical tests performed in Figure 2A, 2B and 2C (which has adjacent normal tissue)

Point-to-point response: Thank you for your helpful suggestion. We have added the statistical tests in Figure 2A (ANOVA test), 2B (ANOVA test), and 2C (paired t-test), and all of the three databases have adjacent normal tissue. We revised the figure legends accordingly.

4. The comment of Reviewer & Prof. Xueer Chen:

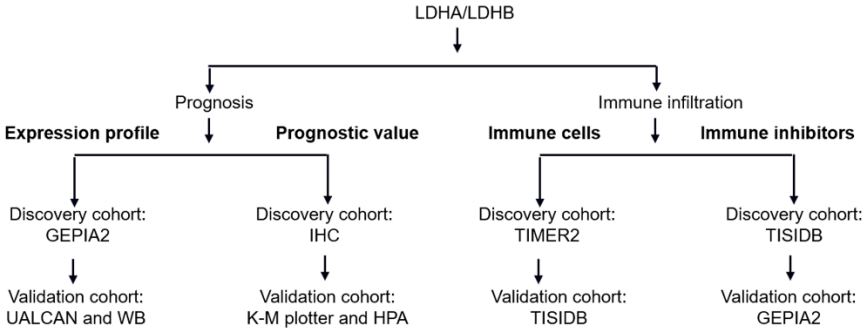
4.1 Basic reporting

4.1.1 figure 1 needs more clear annotation, "discovery cohort", "validation cohort" need to be more specific with data resources and cohort sizes, the current annotation might confuse the readers to think that the same "discovery" and "validation" cohorts

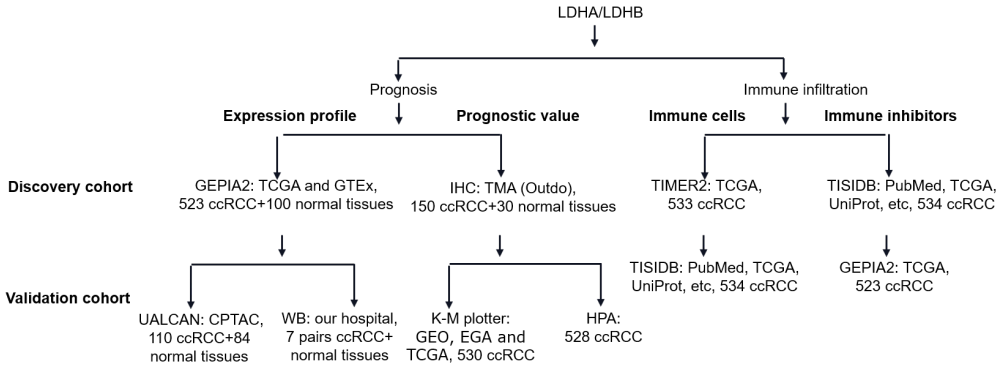
were used across the four tasks (expression profile, prognostic value, immune cells, immune inhibitors).

Point-to-point response: Thank you for your helpful suggestion. We have re-organized the Figure 1 in the revised manuscript, which included more specific information with data resources and cohort sizes.

former:



Later:



4.1.2 please check why there are two same plots for “purity” and LDHB expression on Figure 4.

Point-to-point response: Thank you for your helpful suggestion. For the symmetry and beauty, we put two same plots for “purity” and LDHB expression on Figure 4. If there is only one plot for “purity”, there are 5 plots in the upper lane and 4 ones in the lower lane. We delete keep one ‘purity’ plot and only keep one plot in the revised manuscript according to your suggestion. Thank you again.

4.1.3 Is the “immunoinhibitors” meaning immune checkpoint inhibitors? Please elaborate the concept. In addition, how did the authors curate the 24 immunoinhibitors, what are the references and is the list comprehensive?

Point-to-point response: Thank you for your helpful suggestion. According to the TISIDB (PMID: 30903160), immunoinhibitors are a kind of immunomodulators, which means immune checkpoint inhibitors. The detailed list of the 24 immunoinhibitors are ADORA2A, BTLA, CD160, CD244, CD274, CD96, CSF1R, CTLA4, HAVCR2, IDO1, IL10, IL10RB, KDR, KIR2DL1, KIR2DL3, LAG3, LGALS9, PDCD1, PDCD1LG2, PVRL2, TGFB1, TGFBR1, TIGIT, and VTCN1, which are collected from Charoentong's study(PMID, 28052254). Thank you again.

4.2 Experimental design

4.2.1 potential overlap of samples across "discovery cohort" and "validation cohort" need to be checked and reported to support the appropriate validation process. It's essential to use non-overlap discovery and validation cohorts for the validation process. If the databases adapted the same or overlapped samples, the validation would not be reliable.

Point-to-point response: Thank you for your helpful suggestion. Yes, we checked the original datasets of the different databases, and found there were no overlap between the databases for prognosis. But for immune infiltration, there are some overlapped samples for TIMER2 and TISIDB. For immune cells, they use different deconvolution methods or algorithms and different cell types, which is reliable. As for immune inhibitors, TISIDB explores tumor immune whereas GEPIA2 detects gene expression, they are relative different and independent algorithms. Thank you again.

4.2.2 For the study of "immune cells", the cell types are not aligned with TIMER2 and TISIDB, how would the authors proceed with the validation process? what conclusion can be drawn if there are no obvious common cell types for these two methods?

Point-to-point response: Thank you for your helpful suggestion. Yes, this is an interesting question. The immune cells types are not aligned with TIMER2 and TISIDB, and there are 8 TIICs in TIMER2 & 28 TILs in TISIDB. Although TISIDB and TIMER2 are originated from different datasets and team, they use different deconvolution methods or algorithms. And both are comprehensive and embrace almost all the tumor related immune cells. In this opinion, we could deduce and

conclude the results. Please let us know if you have any additional concerns or suggestions. Thank you again for your helpful feedback.

4.3 Validity of the findings

4.3.1 the authors have shown the differential expression of LDHA/LDHB among tumor and normal samples, however, the conclusions and potential mechanisms of association of LDHA/LDHB with immune infiltration (immune cells and immune inhibitors) are not well explained and described.

Point-to-point response: Thank you for your helpful suggestion. Yes, this is a good idea. We have tried to explore the relationship and potential mechanisms of association of LDHA/LDHB with immune infiltration using STRING and DAVID databases, and found there was no direct interaction between them (See response to Shruti Singh Kakan). In the discussion, we also denote this issue (shortcoming), and we hope to clarify this issue in the further exploration. Thank you again.

4.3.2 the datasets used for discovery and validation of the four tasks (expression, prognostic, immune cells and immune inhibitors) are not well described and can be ambiguous for the readers.

Point-to-point response: Thank you for your helpful suggestion. According to your esteemed suggestion, we have rephrased Figure 1 and made it clear to the readers (See 4.1.1). Thank you again.