nature portfolio

Peer Review File

The protein circPETH-147aa regulates metabolic reprogramming in hepatocellular carcinoma cells to remodel immunosuppressive microenvironment

Corresponding Author: Professor Hong Wu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Lan et al show that a circular RNA from tumor-associated macrophages (TAM) reprograms the metabolism of heptacellular carcinoma cells (HCC). The authors demonstrate that the circular RNA, circPETH, is excreted from TAM via extracellular vesicles, and is taken up by HCC. Translation of circPETH in a m6A-dependent manner produces a microprotein, circPETH-147aa. The protein alters the phenotype of HCC, reprogramming cellular metabolism and impairing anti-HCC treatment. The authors screened a small molecule library and identified the xanthone compound norathyriol as having the ability to reverse circPETH-147aa-dependent metabolic reprogramming and increasing the efficacy of anti-PD1 therapy. The ability to target metabolic reprogramming to overcome drug resistance is an exciting and promising discovery. The findings will be of broad interest to the field.

The manuscript reflects a huge amount of effort by the authors – 7 main figures and 33 supplemental figures and tables, most of which are multi-panel – for which the authors should be congratulated. Overall, the experiments are well done with appropriate controls. Critical questions are approached using multiple experimental systems and the results seem robust. However, several critical controls are missing that need to be addressed prior to publication.

Major issues:
1) SPR binding experiments for protein-protein interactions (Main Figure 3a-d, Extended data figures 14e,f, 15d,e, 16f-l , 17d,e, 21d). The sensorgram for the binding between proteins is not fully shown. This is curious, as full sensorgrams are shown for the binding between circPETH-147aa and norathyriol (Fig 5c). Further, the association phase between the proteins, such as circPETH-147aa and HuR (Fig 4e), do not reach plateau and dissociation phase shows almost no loss of binding. The full sensorgram and kinetic modeling of the binding must be shown to ensure that the interaction is specific and not due to non-specific binding or protein precipitation. Binding affinities should be expressed with SEM to describe the variability of these parameters.

2) F-actin interaction experiments (ED Figure 11) – These experiments are not described in the methods and materials. Without further details on the F-actin immunoprecipitation assays, it is difficult to assess if the interaction is a valid measurement of interaction with polymerized actin. Further, the quality of the images of what I assume are phalloidin-labeled F-actin filaments are not of sufficient magnification and resolution.

3) Norathyriol studies (Figure 5-7, Extended data figures 23-25). The authors screened and identified norathyriol, a xanthone compound, as a compound targeting the MEG pocket of circPETH-147aa. The experiments on norathyriol are inadequately described in the results section. The key question is whether the impact of norathyriol on tumor growth in vivo is dependent on circPETH-147 or if it is due to a circPETH-147-independent effect; xanthone compounds have been described to have multiple anti-cancer properties. For the in vivo experiments (Fig 5e,f), the impact of a non-active norathyriol analog or a non-norathyriol binding circPETH-147aa mutant should be determined. For example, the impact of norathyriol treatment on OE-circPETH MEG/3A tumor growth (Fig 5e,f) would show if the drug has non-specific effects on tumor growth.

4) Scientific communication: The huge volume of data presented in this manuscript makes it hard to read and understand. Key findings are not adequately discussed, and key data is often found in supplemental figures. The authors should

consider splitting the manuscript into multiple manuscripts that allow for a simpler and clearer message.

Minor issues:
1) The manuscript would benefit from editorial assistance to correct grammatical errors and improve the fluidity of the manuscript.

2) Amino acid measurements: Measurements of amino acids were performed to address whether the either siRNA knockdown of SLC43A2 or genomic editing to knockout circPETH-147aa causes dysregulation of amino acid homeostasis. These assays are not described in the methods and materials. Please add this description in sufficient detail to assess these experiments.

Reviewer #2

(Remarks to the Author)
It's a well done research about circRNA-encoded protein in HCC. circPETH-147aa is encoded by circPETH in a m6A-driven manner and promotes PKM2-catalyzed ALDOA-S36 phosphorylation via MEG pocket. small-molecule Norathyriol acts as an effective inhibitor that targets the MEG pocket on circPETH-147aa surface.

Some issues are proposed before publishing:
1. In methods, ''Norathyriol (HY-N1029, MCE) was intraperitoneally injected (5 mg/kg or 10 mg/kg, once daily for two weeks)'', 5 or 10 is used?
2. Why the Norathyriol is identified as the inhibitor?
3. In Extended Data Fig. 22: in the b, ''Boxes indicate the areas with higher magnification shown at the upper right corner. Scale bar, 50 μm.'' What do you want to show? CD8+ T and cancer cells?
In the c, the coculture image is too simple. Is it a transwell or directly co-culture?
4. In fig 5e, 5f, some of the mice seems skinny. What's the time of duration? While, in fig 6a, 6b, it's well.
5. New references about circRNA biogenesis and m6A are suggested in citation, e.g. PMID: 31570856
6, in fig 6c, the coculture with CD8+ T is in vivo or in vitro? Homo genus or Mouse species?
7. Fig 7e, the exosome and lactate are vital elements. What's the data of them in the Figures?
Or, extracellular vesicles= exosome?
8. this results 1st part is too long to read. ''TAMs promote the invasion, migration and aerobic glycolysis of HCC cells via the action of EV-loaded circPETH''.
9. Whare the m6A is installed to circPETH-147aa? The HCC cells cytoplasm?

Reviewer #3

(Remarks to the Author)
The paper, titled 'The Novel Protein circPETH-147aa Regulates Metabolic Reprogramming in Hepatocellular Carcinoma Cells to Remodel the Immunosuppressive Microenvironment,' provides a comprehensive description of an immunoregulatory pathway between macrophages in the tumor environment and tumor cells. It is supported by eight main figures and over twenty supplemental complex figures. The research focus on the mechanism, from the packaging of circular RNA in vesicles to the action of a molecule that inhibits protein synthesis from the circular RNA, ultimately preventing tumor cell proliferation and migration.

- What are the noteworthy results?

Description of the circPETH RNA, described as packaged in extracellular vesicles released by tumor associated macrophages.
Structure and function of the newly described protein.
Immunosuppressive mechanism of the protein.
Testing of an inhibitory drug targeting circpeth-147aa: in vivo efficacy and combination therapy with anti-pdl1

- Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.

They are the first to describe both this particular circRNA, and the protein transcribed for it, including structure and function. It has been described previously a hsa_circRNA_001587 that upregulates a solute carrier protein, SLC4A4, through miRNA mechanism (32878470). The one exposed in this article does not required miRNA, but recruit a RNA binding protein. The work is not only useful from the cell biology perspective, but also provide a new target for antitumoral treatment.

- Does the work support the conclusions and claims, or is additional evidence needed?

The article provides enough data and support of the conclusions.
Regarding EV packaging, additional tests can be performed to demonstrate that the circRNA is indeed associated with the vesicular fraction of the EV preparation.

This can include media fractionation by size exclusion chromatography to verify that circRNA is primarily present in the fractions associated with vesicles.
Additionally, isolating vesicles using CD63, CD81, or CD9 antibody capture and confirming that circRNA co-purifies with these vesicles would be informative.
It could be interesting to lyse the vesicles with detergent and then expose tumoral cells to the treated EVs to observe if the disruption of the vesicles affects the observed effects. Referring to the MISEV guidelines (10.1002/jev2.12404; 10.1080/20013078.2018.1535750) is crucial when publishing articles related to EVs.

- Are there any flaws in the data analysis, interpretation and conclusions? - Do these prohibit publication or require revision?

Data seems to be appropriate.

- Is the methodology sound? Does the work meet the expected standards in your field?

Techniques were performed according to the expected standards.

- Is there enough detail provided in the methods for the work to be reproduced?

Data analysis seems to be appropriate, although certain information may be missing in some figures.
For instance, number of replicates in most of the experiments. How many animals were employed for in vivo studies? Also some general description of how the antibody was generated will be useful.
Regarding EV treatment, which amount of EVs were employed to treat cells?
It is described how to calculate glycolytic level through sea-horse, but apparently this value is not presented. However, there are not protocol about how to calculate relative glucose consumption or relative lactate production.

Reviewer #4

(Remarks to the Author)
The report describes the discovery of a novel small-molecule Norathyriol as an inhibitor that targets the MEG pocket on circPETH-147aa. My report includes only opinion on the molecular modelling and virtual screening activities involved in discovery of this ligand.
The MEG pocket on circPETH-147aa was targeted for virtual screening to identify candidates which might inhibit the circPETH-147aa–protein interaction. MOE sitefinder was used to identify suitable pockets on the surface of the MEG pocket. Although the pockets would appear to demonstrate the potential for occupancy by small molecules, it would be helpful to include additional detail of this (druggability metrics and scores) to convince the reader of the druggability of the identified pockets. An alternative approach would be to use the SiteMap tool within Schrodinger. The authors would appear to have access to this software so are they able to support the findings by additionally applying this methodology to their validation?
The virtual screening and compound filtering activities using the Schrodinger software appear sensible and robust. Is it possible for the authors to clarify which specific TargetMol library was used? Was it a general screening library, or is there a particular focus to the screening set? A number of candidate compounds were selected for SPR screening. The discovered compound – Norathyriol – is a polyphenol which is an example of a class of compound which has the potential for complex polypharmacology. I'm not qualified to comment on the downstream experimental data to further critique on this comment.
The protein modelling activities through use of the Z-dock methodology appear robust and would appear to support the findings of the experimental work.
The figures are well crafted and accompanied by suitably detailed legends. The experimental details (methods) are suitably descriptive

Reviewer #5

(Remarks to the Author)
This study reports on the finding that circPETH-147aa regulates metabolic reprogramming and TME by promoting PKM2-catalyzed ALDOA-S36 phosphorylation and elevating HuR-dependent SLC43A2 mRNA stability, and has potential to be a new therapeutic strategy to overcome HCC resistance to ICB therapies. While this observation is interesting and has significance for the treatment of HCC. However, there are several points at which the data supplied is not strong enough for the conclusions made.
Major points:
1. Almost all the animal experiments are using human HCC cell line, such as Hep3B and Huh7 in immunodeficient mice. This limitation weakens the conclusions regarding circPETH-147aa's function on tumor microenvironment.
2. While the authors have demonstrated a correlation between circPETH expression and HCC patient outcomes (Extended Data Fig. 3), the relationship between circPETH and ICI response, as well as immune cell infiltration in HCC patients, remains to be investigated to support the clinical significance by targeting circPETH-147aa on remodeling immunosuppressive TME in HCC.
3. One of their major findings is that circPETH-147aa impairs anti-HCC immunity by inducing methionine and leucine deficiency in cytotoxic CD8+ T cells. However, flow cytometry analysis by co-culturing tumor cell with T cell is too rough to detect T cell anti-tumor activity because it cannot reflect specific anti-tumor response. Tumor-specific antigen, such as OVA, should be established on tumor cells, or at least analysis immune profile in TME by in-vivo HCC mouse model.
4. As mentioned in the comment above, the authors performed in-vitro co-culture experiments to verify that Norathyriol

boosts cytotoxic CD8+ T-cell function. The lack of in-vivo experiments making it hard to support this conclusion.
5. The authors examined the relative expression levels of several markers (such as PD-L1, CTLA4, IFN-r, and GZMB) on CD8+ T cells using RT-PCR. However, flow cytometry is a more appropriate technique for detecting specific cell populations, such as PD-L1+ CD8+ T cells, as it allows for the quantification of surface marker expression levels, which are more directly relevant to their functional roles in the tumor microenvironment (TME).

Minor points:
1. Are these findings specific for HCC? The authors should show data to address this to increase clinical relevance.
2. The gating strategies for flow cytometry analysis should be provided in the manuscript or supplementary materials.
3. The resolution of Extended Data Fig. 19b should be improved for better visualization and interpretation.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have answered all of my questions. The manuscript is acceptable for publication.

Reviewer #2

(Remarks to the Author)
The authors' answers completely resolved my doubts. So I am satisfied with that answer. Next, I support publication.

Reviewer #3

(Remarks to the Author)
The authors have addressed all the questions I raised and have carefully considered each of my comments in their responses. They provided detailed clarifications where needed and made adjustments to the manuscript accordingly. I am satisfied with the revisions made and believe the manuscript is easier to understand now. Thank you for facilitating this exchange.

Reviewer #4

(Remarks to the Author)
Thank you for the response to my queries associated with the virtual screening. I'm happy with the responses and make no further comment or requests of the authors

Reviewer #5

(Remarks to the Author)
I appreciate the revisions to the manuscript resubmitted by the authors. They have extensively revised and clarified the major questions raised by the reviewers, and have provided new data that strengthens their work.

Point-by-point response to the reviewer comments

Dr. Reviewers

We would like to thank you for your valuable comments, which are essential for us to further improve the quality and accuracy of our manuscript. We have carefully read the concerns and suggestions. Those comments are very helpful for improving our work.

In this revised manuscript, with all the useful comments, we have made significant revisions and added all necessary experiments to make the contents and conclusions more comprehensive. Please find below a list of our detailed responses to all the comments and corrections in the revised manuscript. All the corrections and changes in the revised manuscript are highlighted in red.

Reviewer reports:

Reviewer #1 (Remarks to the Author): expert in glycolytic metabolism

In this manuscript, Lan et al show that a circular RNA from tumor-associated macrophages (TAM) reprograms the metabolism of heptacellular carcinoma cells (HCC). The authors demonstrate that the circular RNA, circPETH, is excreted from TAM via extracellular vesicles, and is taken up by HCC. Translation of circPETH in a m6A-dependent manner produces a microprotein, circPETH-147aa. The protein alters the phenotype of HCC, reprogramming cellular metabolism and impairing anti-HCC treatment. The authors screened a small molecule library and identified the xanthone compound norathyriol as having the ability to reverse circPETH-147aa-dependent metabolic reprogramming and increasing the efficacy of anti-PD1 therapy. The ability to target metabolic reprogramming to overcome drug resistance is an exciting and promising discovery. The findings will be of broad interest to the field.

The manuscript reflects a huge amount of effort by the authors – 7 main figures and 33 supplemental figures and tables, most of which are multi-panel – for which the

authors should be congratulated. Overall, the experiments are well done with appropriate controls. Critical questions are approached using multiple experimental systems and the results seem robust. However, several critical controls are missing that need to be addressed prior to publication.

Response:

Thank you for your recognition of our work.

Major issues:

1) SPR binding experiments for protein-protein interactions (Main Figure 3a-d, Extended data figures 14e,f, 15d,e, 16f-l , 17d,e, 21d). The sensorgram for the binding between proteins is not fully shown. This is curious, as full sensorgrams are shown for the binding between circPETH-147aa and norathyriol (Fig 5c). Further, the association phase between the proteins, such as circPETH-147aa and HuR (Fig 4e), do not reach plateau and dissociation phase shows almost no loss of binding. The full sensorgram and kinetic modeling of the binding must be shown to ensure that the interaction is specific and not due to non-specific binding or protein precipitation. Binding affinities should be expressed with SEM to describe the variability of these parameters.

Response:

Thank you for your kind suggestion. We have revised the sensorgrams for the bindings between proteins (Main Figures 3a-d, 4e, Extended data figures 14e,f, 15d,e, 16f-l , 17d,e, 21d). The sensorgrams for all bindings between proteins have been fully shown in figures. The protein-protein interaction is significantly slower than that between protein and small-molecule. Therefore, the plateau was relatively difficult to be reached in the association phase between the proteins and the dissociation phase was associated with a slow loss of binding, rather than no loss of binding, which was consistent with previously reported studies[1-3]. Binding affinities have been expressed with SEM. All the corresponding full Sensorgrams and Bindings to reference have been provided in in “Source Data” file according to editorial requests.

1. Lu C, Meng C, Li Y, et al. A probe for NIR-II imaging and multimodal analysis of early

Alzheimer's disease by targeting CTGF. ***Nat Commun.*** 2024;15(1):5000. (Figure 3a)

[REDACTED]

2. Dai L, Zheng T, Xu K, et al. A Universal Design of Betacoronavirus Vaccines against COVID-19, MERS, and SARS. ***Cell.*** 2020;182(3):722-733.e11. (Figure 3c, 4c)

[REDACTED]

3. Xu K, Gao P, Liu S, et al. Protective prototype-Beta and Delta-Omicron chimeric RBD-dimer vaccines against SARS-CoV-2. ***Cell.*** 2022;185(13):2265-2278.e14. (Figure S2)

[REDACTED]

2) F-actin interaction experiments (ED Figure 11) – These experiments are not described in the methods and materials. Without further details on the F-actin immunoprecipitation assays, it is difficult to assess if the interaction is a valid measurement of interaction with polymerized actin. Further, the quality of the images of what I assume are phalloidin-labeled F-actin filaments are not of sufficient magnification and resolution.

Response:

Thank you for your kind suggestion. The details of the F-actin immunoprecipitation assays have been presented in the methods. Below is the newly added text.

Page 43: "As for the F-actin immunoprecipitation assays, cells were collected and lysed with IP lysis buffer (100 mM NaCl, 0.5 mM EDTA, 20 mM Tris-HCl, pH7.4, 0.5% NP-40), and immunoprecipitated with 1 μg anti-LATS antibody (Cell Signaling

Technology, cat# 3477) at 4°C overnight. Then lysates were incubated with protein A Sepharose (GE Healthcare, cat# 17-0963-03) or protein G agarose (Millipore, cat# 16-266) for 2 hours followed by 3 washes using binding buffer (150 mM NaCl, 0.5 mM EDTA, 20 mM Tris-HCl, pH7.4, 0.5% NP-40). Finally, the beads were eluted with SDS-PAGE loading buffer and the resultant elutes were subjected to immunoblotting analysis."

IP lysis buffer exerts little influence on the depolymerization of F-actin. More importantly, cells were treated with Latrunculin B (Lat. B) to ensure that there was no interaction between LATS1 and depolymerized actin (Extended data figure 11g-i). In addition, the images of phalloidin-labeled F-actin filaments have provided with high resolution (Extended data figure 11j,k and Extended data figure 12a,b).

3) Norathyriol studies (Figure 5-7, Extended data figures 23-25). The authors screened and identified norathyriol, a xanthone compound, as a compound targeting the MEG pocket of circPETH-147aa. The experiments on norathyriol are inadequately described in the results section. The key question is whether the impact of norathyriol on tumor growth in vivo is dependent on circPETH-147 or if it is due to a circPETH-147-independent effect; xanthone compounds have been described to have multiple anti-cancer properties. For the in vivo experiments (Fig 5e,f), the impact of a non-active norathyriol analog or a non-norathyriol binding circPETH-147aa mutant should be determined. For example, the impact of norathyriol treatment on OE-circPETH MEG/3A tumor growth (Fig 5e,f) would show if the drug has non-specific effects on tumor growth.

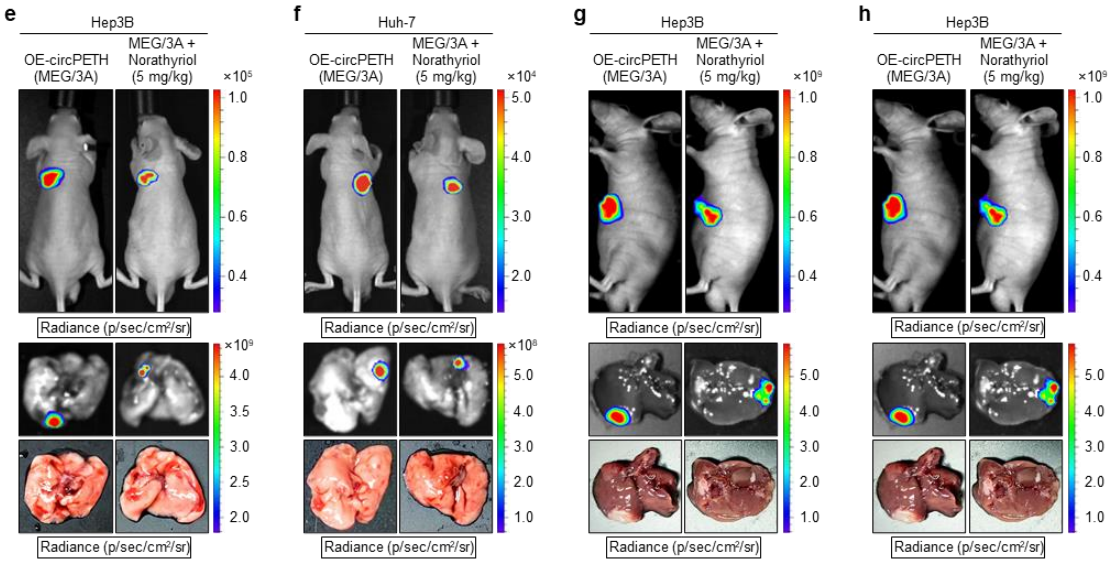
Response:

Thank you for your kind suggestion. Indeed, xanthone compounds have been reported to have multiple anti-cancer properties. The in vivo experiments had the limitation that you mentioned. Therefore, we assessed the impact of Norathyriol treatment on OE-circPETH MEG/3A tumor growth. The results showed that following Norathyriol treatment, OE-circPETH MEG/3A group presented with reduced bioluminescence intensity and decreased fluorescence intensity in the lungs (Extended data figure

26e,f). In addition, the intrahepatic bioluminescence intensity and fluorescence intensity were declined after Norathyriol treatment (Extended data figure 26g,h). Indeed, apart from suppressing the function of the MEG pocket of circPETH-147aa, Norathyriol exerted non-specific effects on HCC growth to some extent. However, we could see that the inhibitory impacts of Norathyriol on OE-circPETH MEG/3A group were considerably lower than that of Norathyriol on OE-circPETH WT group. Therefore, the inhibition of Norathyriol on HCC was mainly dependent on circPETH-147. Below is the newly added text.

Page 24, 25: "Notably, it has been reported that norathyriol has multiple anticancer activities. Indeed, in addition to suppressing the function of the MEG pocket of circPETH-147aa, norathyriol had nonspecific effects on HCC metastasis (Extended Data Fig. 26e-h). However, the inhibitory effects of norathyriol in the OE-circPETH MEG/3A group were considerably weaker than the inhibitory effects of norathyriol in the OE-circPETH WT group. Overall, the inhibitory effect of norathyriol on HCC cells was dependent mainly on circPETH-147."

The data were also shown as below:



4) Scientific communication: The huge volume of data presented in this manuscript makes it hard to read and understand. Key findings are not adequately discussed, and

key data is often found in supplemental figures. The authors should consider splitting the manuscript into multiple manuscripts that allow for a simpler and clearer message.

Response:

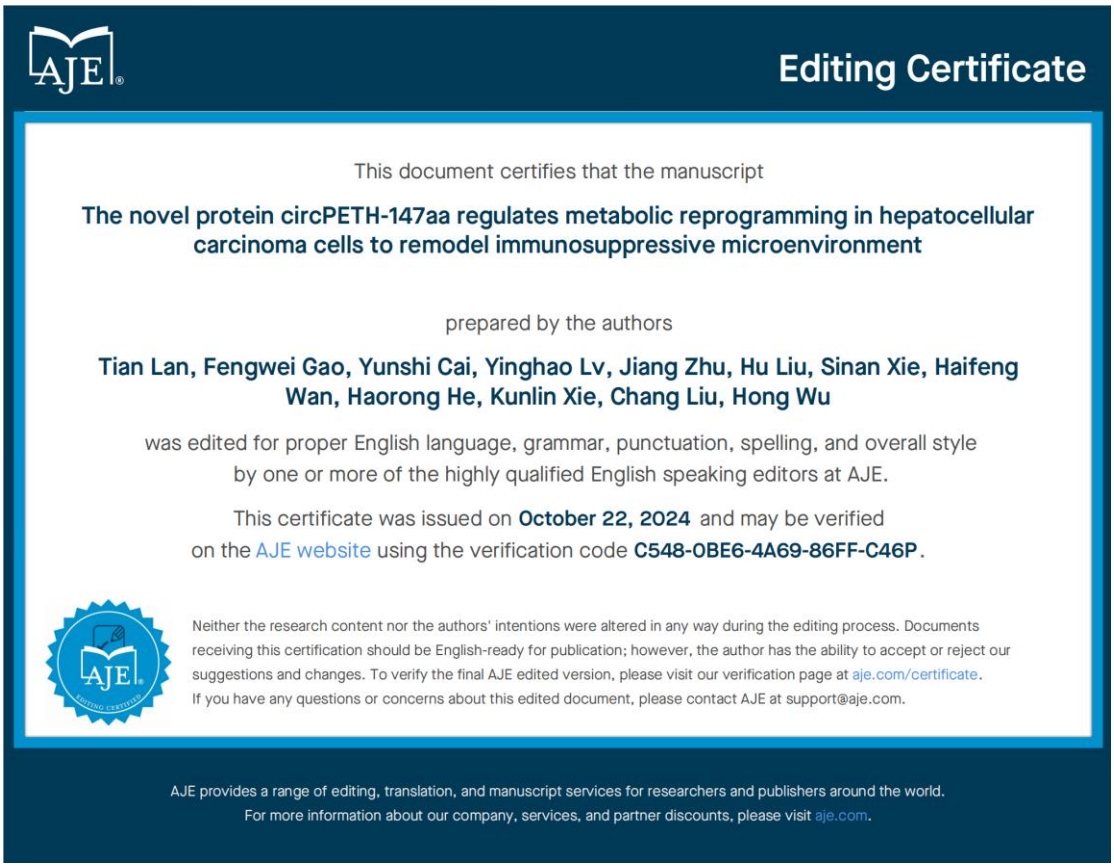
Thank you for your kind suggestion. The biogenesis, function, mechanism and validation of circPETH, and its newly-identified small-molecule inhibitor were all investigated in our manuscript. We aimed to perform a relatively complete research process for a novel molecular target in a disease model from its discovery to "preliminary drug". Therefore, the data was kind of huge. In order to better understand, we have rediscussed the key findings and reorganized the key data. Actually, we have split our overall research work into several parts. Apart from this submitted manuscript, drug safety, pharmacodynamics and pharmacokinetics of small-molecule compound and big animal experiments were involved in other research contents.

Minor issues:

1) The manuscript would benefit from editorial assistance to correct grammatical errors and improve the fluidity of the manuscript.

Response:

Thank you for your kind suggestion. We have carefully checked and modified throughout the manuscript as much as possible. Importantly, the manuscript has been edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of the highly qualified English-speaking editors at American Journal Experts (AJE), and the English Editing Certificate has been provided in Supplementary information and also show as below:



2) Amino acid measurements: Measurements of amino acids were performed to address whether the either siRNA knockdown of SLC43A2 or genomic editing to knockout circPETH-147aa causes dysregulation of amino acid homeostasis. These assays are not described in the methods and materials. Please add this description in sufficient detail to assess these experiments.

Response:

Thank you for your kind suggestion. The description of amino acids quantifications has been added in the methods. Below is the newly added text.

Page 43, 44: "Methionine quantification

The methionine concentration in culture medium was measured using Methionine Assay Kit (Abcam, ab234041). The medium was collected and diluted to 100 µl with PBS, and then 2 µl of Sample Clean-up mix was added and incubated at 37 °C for 30 min. Samples were filtered through a 10 kDa spin column (10,000 × g, 4 °C, 10 min), and the ultrafiltrate was retained. For each sample, two parallel wells were prepared, one for the determination of methionine and one for a sample background

control. Totally, 5 µl ultrafiltrate and 20 µl detection buffer were added to each well. Then 25 µl reaction mix and 25 µl background control mix were added to their parallel sample wells, respectively. Meanwhile, 100 µM methionine standard was used to prepare the standard curve. Fluorescence was read in endpoint mode (Ex/Em = 535/587 nm) after 30 min of incubation of the plate at 37 °C. The absolute methionine concentration was calculated for each sample using the standard curve.

Leucine quantification

The quantification of leucine in culture medium was conducted using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (API3200, AB Sciex). A mass spectrometry conjugated with an electrospray ionization (ESI) interface was used to produce positive ions. Optimized mass parameters of leucine were determined by infusing each solution of the compounds at 1 ng/mL into the mass spectrometer in the positive ion mode at 10 µL/min. The chromatographic separation of leucine in culture medium was conducted with Gemini-NX 3µ C18 110 A New column 50 × 2.0 mm (656065-14) using an isocratic elution condition of 0.1% formic acid in water and methanol (50:50, v/v) at 0.3 mL/min and 40 °C using the Agilent 1200 series HPLC system (Agilent Technologies)."

Reviewer #2 (Remarks to the Author): expert in circRNAs, m6A, some glycolysis

It's a well done research about circRNA-encoded protein in HCC. circPETH-147aa is encoded by circPETH in a m6A-driven manner and promotes PKM2-catalyzed ALDOA-S36 phosphorylation via MEG pocket. small-molecule Norathyriol acts as an effective inhibitor that targets the MEG pocket on circPETH-147aa surface.

Response:

Thank you for your recognition of our work.

Some issues are proposed before publishing:

1. In methods, '' Norathyriol (HY-N1029, MCE) was intraperitoneally injected (5 mg/kg or 10 mg/kg, once daily for two weeks)'' , 5 or 10 is used?

Response:

Thank you for your kind suggestion. The effects of Norathyriol with two different concentrations (5 mg/kg and 10 mg/kg) on the lung and intrahepatic metastasis of HCC cells were both determined in Figure 5e,f and Figure 6a,b. Overall, Norathyriol (HY-N1029, MCE) was intraperitoneally injected (5 mg/kg and 10 mg/kg for two different concentration groups, respectively, once daily for two weeks).

2. Why the Norathyriol is identified as the inhibitor?

Response:

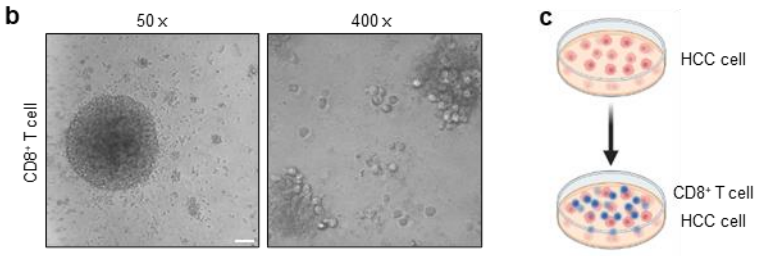
Thank you for your kind suggestion. Our work showed that circPETH-147aa promoted the aerobic glycolysis and metastasis of HCC cells, and impaired anti-HCC immunity by the interactions between the MEG pocket on its surface and other targets. We identified Norathyriol with the highest affinity for circPETH-147aa via a series of assays, which could occupy the MEG pocket and subsequently disrupted the bindings of circPETH-147aa to effector proteins. Therefore, Norathyriol was identified as the potential inhibitor of circPETH-147aa.

3. In Extended Data Fig. 22: in the b, ‘’ Boxes indicate the areas with higher magnification shown at the upper right corner. Scale bar, 50 μm.’’ What do you want to show? CD8+ T and cancer cells?

In the c, the coculture image is too simple. Is it a transwell or directly co-culture?

Response:

Thank you for your kind suggestion. b showed the phase-contrast micrographs of activated and amplified CD8+ T cells by IL-2, anti-CD3 and anti-CD28 antibodies with different magnifications. As for c, HCC cells were directly co-cultured with CD8+ T cells without transwell. We have revised the figures and its corresponding legends. The data were also shown as below:



4. In fig 5e, 5f, some of the mice seems skinny. What’s the time of duration? While, in fig 6a, 6b, it’s well.

Response:

Thank you for your kind suggestion. The duration was 6 weeks. As we got a lot of mouse groups in this work, mice were purchased in batches, which resulted in differences in body weight and growth. In addition, the shooting angle in Fig. 5e,f made these differences seem bigger. Generally, the main reason why nude mice bearing tumor cells present skinny is the cachexia caused by large tumor load, which also leads to low vitality and loss of appetite. However, mice in our work generally showed high vitality and appetite. Therefore, mice data were reliable in this work.

5. New references about circRNA biogenesis and m6A are suggested in citation, e.g. PMID: 31570856

Response:

Thank you for your kind suggestion. We have added two new references (PMID: 31570856 and PMID: 31887551) about circRNA biogenesis and m6A in citation.

1. Zhao, W. *et al.* Splicing factor derived circular RNA circUHRF1 accelerates oral squamous cell carcinoma tumorigenesis via feedback loop. ***Cell death and differentiation*** **27**, 919-933 (2020).
2. Zhao, W. *et al.* Epigenetic Regulation of m(6)A Modifications in Human Cancer. ***Molecular therapy. Nucleic acids*** **19**, 405-412 (2020).

6, in fig 6c, the coculture with CD8+ T is in vivo or in vitro? Homo genus or Mouse species?

Response:

Thank you for your kind suggestion. The coculture with $CD8^+$ T isolated from human peripheral blood was in vitro.

7. Fig 7e, the exosome and lactate are vital elements. What’ s the data of them in the Figures? Or, extracellular vesicles= exosome?

Response:

Thank you for your kind suggestion. The data of exosomes were presented in Extended Figures, especially in Extended Data Fig. 2. The association between circPETH, ADAR1 and lactate was showed in Extended Data Fig. 7c-g. In this work, extracellular vesicles were equal to exosomes from the perspectives of particle diameter and biomarker expressions.

8. this results 1st part is too long to read. ‘’ TAMs promote the invasion, migration and aerobic glycolysis of HCC cells via the action of EV-loaded circPETH’’ .

Response:

Thank you for your kind suggestion. We have divided the 1st part into two sections.

Section 1: “TAMs promote the HCC cell invasion, migration and aerobic glycolysis via EV-loaded circPETH” (**Page 4-7**)

Section 2: “ADAR1 inhibits the circularization of circPETH, and the loss of introns leads to the knockout of circPETH” (**Page 7-10**)

9. Whare the m6A is installed to circPETH-147aa? The HCC cells cytoplasm?

Response:

Thank you for your kind suggestion. FISH revealed that circPETH was predominantly located in the cytoplasm of both HCC cells and macrophages. Its m6A installation was performed in the cytoplasm and drove the translation of circPETH into circPETH-147aa. While, subcellular protein fractionation and IF revealed that the expression of circPETH-147aa protein was higher in the nucleus than in the cytoplasm.

Reviewer #3 (Remarks to the Author): expert in EVs, metabolomics

The paper, titled 'The Novel Protein circPETH-147aa Regulates Metabolic Reprogramming in Hepatocellular Carcinoma Cells to Remodel the Immunosuppressive Microenvironment,' provides a comprehensive description of an immunoregulatory pathway between macrophages in the tumor environment and tumor cells. It is supported by eight main figures and over twenty supplemental complex figures. The research focus on the mechanism, from the packaging of circular RNA in vesicles to the action of a molecule that inhibits protein synthesis from the circular RNA, ultimately preventing tumor cell proliferation and migration.

- What are the noteworthy results?

Description of the circPETH RNA, described as packaged in extracellular vesicles released by tumor associated macrophages.

Structure and function of the newly described protein.

Immunosuppressive mechanism of the protein.

Testing of an inhibitory drug targeting circpeth-147aa: in vivo efficacy and combination therapy with anti-pdl1

Response:

Thank you for your recognition of our work.

- Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.

They are the first to describe both this particular circRNA, and the protein transcribed for it, including structure and function. It has been described previously a hsa_circRNA_001587 that upregulates a solute carrier protein, SLC4A4, through miRNA mechanism (32878470). The one exposed in this article does not required miRNA, but recruit a RNA binding protein. The work is not only useful from the cell biology perspective, but also provide a new target for antitumoral treatment.

Response:

Thank you for your recognition of our work.

- Does the work support the conclusions and claims, or is additional evidence needed?

The article provides enough data and support of the conclusions.

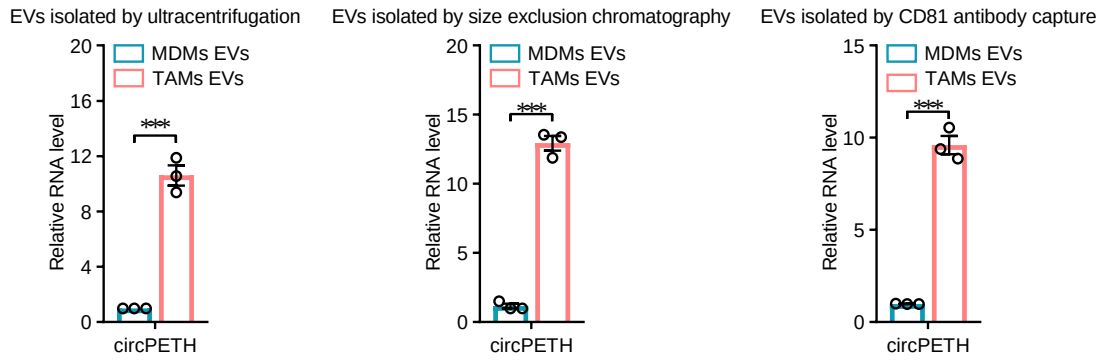
Regarding EV packaging, additional tests can be performed to demonstrate that the circRNA is indeed associated with the vesicular fraction of the EV preparation.

This can include media fractionation by size exclusion chromatography to verify that circRNA is primarily present in the fractions associated with vesicles.

Additionally, isolating vesicles using CD63, CD81, or CD9 antibody capture and confirming that circRNA co-purifies with these vesicles would be informative.

Response:

Thank you for your kind suggestion. Apart form ultracentrifugation, EVs have also been isolated by size exclusion chromatography and CD81 antibody capture. The data for circPETH expression in EVs isolated by three different methods were shown as below:

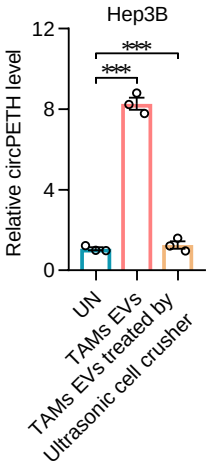


The data were roughly equivalent, showing that circPETH was indeed associated with the vesicular fraction of the EV preparation.

It could be interesting to lyse the vesicles with detergent and then expose tumoral cells to the treated EVs to observe if the disruption of the vesicles affects the observed effects. Referring to the MISEV guidelines (10.1002/jev2.12404; 10.1080/20013078.2018.1535750) is crucial when publishing articles related to EVs.

Response:

Thank you for your kind suggestion. Considering the toxicity of detergents, we used ultrasonic cell crusher to lyse the EVs from TAMs and then exposed them to HCC cells. The results showed that the significant increase of circPETH expression in HCC cells that had been induced by coculture with TAMs EVs was abrogated by the treatment of ultrasonic crushing to EVs. In other words, the disruption of EVs abolished the transmission of circPETH from TAMs to HCC cells.



- Are there any flaws in the data analysis, interpretation and conclusions? - Do these prohibit publication or require revision?

Data seems to be appropriate.

Response:

Thank you for your recognition of our work.

- Is the methodology sound? Does the work meet the expected standards in your field?

Techniques were performed according to the expected standards.

Response:

Thank you for your recognition of our work.

- Is there enough detail provided in the methods for the work to be reproduced?

Data analysis seems to be appropriate, although certain information may be missing in some figures.

For instance, number of replicates in most of the experiments. How many animals were employed for in vivo studies? Also some general description of how the antibody was generated will be useful.

Response:

Thank you for your kind suggestion. We have described the number of replicates for each experiment and the number of animals for each group, and all exact P values of

statistical analysis in figure legends and extended figure legends. Overall, 260 mice were employed for in vivo studies. Antibodies used in this work were presented in Supplementary Table 9. As for the generation of the highly specific antibody against the 28-aa tail of circPETH-147aa, we have provided more details in the section of Methods. Below is the newly added text.

Page 40, 41: "Female BALB/c mice aged 6 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and were immunized subcutaneously 3 times at 10-d intervals with 100 μg purified recombinant 28-aa tail of circPETH-147aa in PBS and Freund's complete adjuvant. The serum titers were analyzed by enzyme-linked immunosorbant assay (ELISA). Mouse spleen cells were isolated and fused with an equivalent number of vital cells of mouse myeloma line SP2/0 by polyethylene glycol (PEG) at a splenocyte-myeloma cell ratio of 10:1, then suspended in HAT selection medium supplemented with 20% FCS (Gibco, Grand Island, NY) and seeded into 96-well plates. To select melted cells, aminopterin was added for 14 days (HAT supplement). Hypoxanthine and thymidine were added to the selection medium for a further period of 7 days. After selection, the remaining hybridoma cells were cultivated in RPMI1640/glutamine medium supplemented with 10% FCS. Hybridoma cells were subjected to selection with the purified recombinant 28-aa tail protein. Three clones of hybridoma with positive activity of 28-aa tail protein binding were obtained. For monoclonal antibody enrichment of antibody production, 1×10^6 hybridoma cells were intraperitoneally injected into a BALB/c mouse. Ascites were collected after 7 days."

Regarding EV treatment, which amount of EVs were employed to treat cells?

Response:

Thank you for your kind suggestion. As for the visualization of EVs incorporation and EVs-encapsulated RNA internalization, 2 μg EVs were employed to treat cells for each experiment.

It is described how to calculate glycolytic level through sea-horse, but apparently this

value is not presented. However, there are not protocol about how to calculate relative glucose consumption or relative lactate production.

Response:

Thank you for your kind suggestion. We have presented the corresponding values of Glycolysis rate and Glycolytic capacity for all ECARs curves. And we have added the details of calculation for relative glucose consumption or relative lactate production in the section of Methods. Below is the newly added text.

Page 37: "Glycolysis rate was calculated as the difference between the ECARs before the injection of oligomycin and the basal ECARs values. Glycolytic capacity was calculated as the difference between the ECARs following the injection of oligomycin and the basal ECARs values. "

Page 37: "The glucose and lactate levels in the medium of negative controls were used for comparison in order to calculate the relative glucose uptake and relative lactate secretion. "

Reviewer #4 (Remarks to the Author): expert in virtual drug screens

The report describes the discovery of a novel small-molecule Norathyriol as an inhibitor that targets the MEG pocket on circPETH-147aa. My report includes only opinion on the molecular modelling and virtual screening activities involved in discovery of this ligand.

The MEG pocket on circPETH-147aa was targeted for virtual screening to identify candidates which might inhibit the circPETH-147aa – protein interaction. MOE sitefinder was used to identify suitable pockets on the surface of the MEG pocket. Although the pockets would appear to demonstrate the potential for occupancy by small molecules, it would be helpful to include additional detail of this (druggability metrics and scores) to convince the reader of the druggability of the identified pockets.

Response:

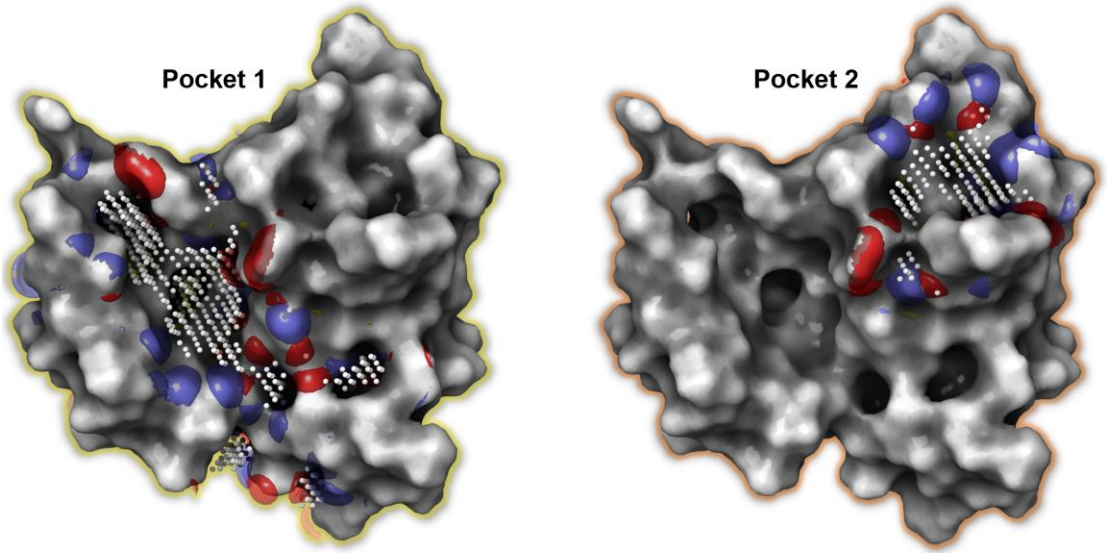
Thank you for your kind suggestion. We identified two pockets on the circPETH-147aa surface by using MOE sitefinder. Pocket 1, which contained 20

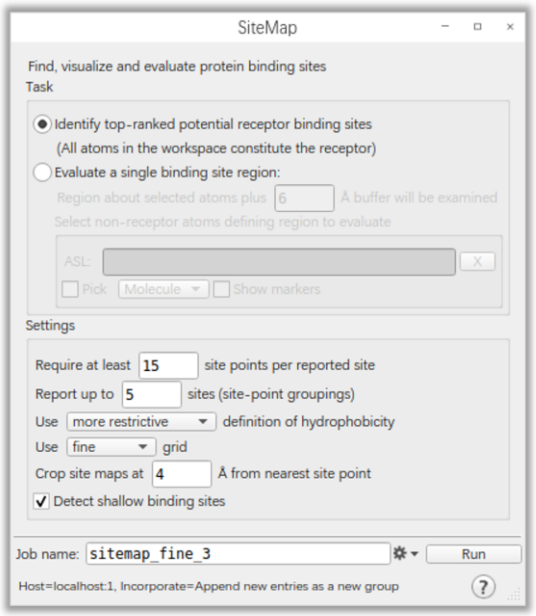
hydrophobic atoms, accommodated up to the compound with 46 atoms. Its Propensity for Ligand Binding (PLB) score was 1.68. As for Pocket 2, it included 22 highly hydrophobic atoms and had a good hydrophobic interaction property, accommodating up to the compound with 43 atoms. Its PLB score was 1.61.

An alternative approach would be to use the SiteMap tool within Schrodinger. The authors would appear to have access to this software so are they able to support the findings by additionally applying this methodology to their validation?

Response:

Thank you for your kind suggestion. The SiteMap tool within Schrodinger has been used to identify suitable pockets on the surface of circPETH-147aa. The results were similar to that generated by MOE sitefinder and were shown as below:





	SiteScore	Dscore
Pocket 1	0.918	1.034
Pocket 2	0.889	1.007

SiteScore

The SiteScore is based on a weighted sum of several of the properties.

A SiteScore of 0.80 has been found to accurately distinguish between drug-binding and non-drug-binding sites.

Druggability Score, Dscore

Dscore uses the same properties as SiteScore but different coefficients.

SiteScore

The SiteScore is based on a weighted sum of several of the properties that are discussed below:

SiteScore = 0.0733 sqrt(n) + 0.6688 e - 0.20 p

where n is the number of site points (capped at 100), e is the enclosure score, and p is the hydrophilic score, and is capped at 1.0 to limit the impact of hydrophilicity in charged and highly polar sites. This score is constructed and calibrated so that the average SiteScore for 157 investigated submicromolar sites is 1.0. Thus, a score of greater than 1 suggests a site of particular promise. A SiteScore of 0.80 has been found to accurately distinguish between drug-binding and non-drug-binding sites

Druggability Score, Dscore

Dscore uses the same properties as SiteScore but different coefficients:

Dscore = 0.094 sqrt(n) + 0.60 e - 0.324 p

For Dscore, the hydrophilic score is not capped. This one of the keys for distinguishing “difficult” and “undruggable” targets from “druggable” ones . The use of different functions for binding-site identification and for classifying druggability is justified because these are different, and sometimes conflicting, tasks. For example, ligands that bind to the PTP1B phosphate pocket with nanomolar, and even subnanomolar, affinity are known . But these highly active ligands have charge structures like those of the natural phosphate substrate and are not drug-like. SiteMap should recognize that such a site can bind ligands tightly but should not rate it as druggable.

The virtual screening and compound filtering activities using the Schrodinger software appear sensible and robust. Is it possible for the authors to clarify which specific TargetMol library was used? Was it a general screening library, or is there a particular focus to the screening set?

Response:

Thank you for your kind suggestion. We used TargetMol (**Bioactive Compound Library**) that contained 40,000 small molecules. It’s a general screening library.

A number of candidate compounds were selected for SPR screening. The discovered compound – Norathyriol – is a polyphenol which is an example of a class of

compound which has the potential for complex polypharmacology. I'm not qualified to comment on the downstream experimental data to further critique on this comment.
The protein modelling activities through use of the Z-dock methodology appear robust and would appear to support the findings of the experimental work.
The figures are well crafted and accompanied by suitably detailed legends. The experimental details (methods) are suitably descriptive

Response:

Thank you for your recognition of our work.

Reviewer #5 (Remarks to the Author): expert in HCC, CD8+ T cells, TAMs

This study reports on the finding that circPETH-147aa regulates metabolic reprogramming and TME by promoting PKM2-catalyzed ALDOA-S36 phosphorylation and elevating HuR-dependent SLC43A2 mRNA stability, and has potential to be a new therapeutic strategy to overcome HCC resistance to ICB therapies. While this observation is interesting and has significance for the treatment of HCC. However, there are several points at which the data supplied is not strong enough for the conclusions made.

Response:

Thank you for your recognition of our work.

Major points:

1. Almost all the animal experiments are using human HCC cell line, such as Hep3B and Huh7 in immunodeficient mice. This limitation weakens the conclusions regarding circPETH-147aa's function on tumor microenvironment.

Response:

Thank you for your kind suggestion. The applications of immunodeficient mouse models and human HCC cell lines were to determine the effects of circPETH-147aa on the metabolic reprogramming and metastasis of HCC. As for tumor microenvironment, we performed the coculture of TAMs and HCC cells and the

coculture of HCC cells and $CD8^+$ T cells to mimic the important substance transmissions and cell-cell interactions in tumor microenvironment. In addition, we established non-immunodeficient C57BL/6J mouse models with implanted Hepa1-6 mouse HCC cells to investigate the impacts of circPETH-147aa on immunotherapy of HCC.

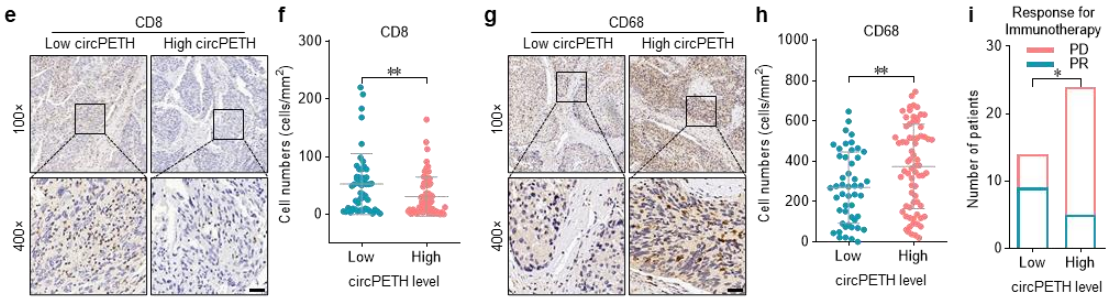
2. While the authors have demonstrated a correlation between circPETH expression and HCC patient outcomes (Extended Data Fig. 3), the relationship between circPETH and ICI response, as well as immune cell infiltration in HCC patients, remains to be investigated to support the clinical significance by targeting circPETH-147aa on remodeling immunosuppressive TME in HCC.

Response:

Thank you for your kind suggestion. We have analyzed the relationship between circPETH and ICI response, as well as immune cell infiltration in HCC patients. Below is the newly added text.

Page 23, 24: "Finally, we assessed the relationship between circPETH expression and the response to ICBs in HCC patients, as well as immune cell infiltration in tumour tissues. The results revealed that patients with high circPETH levels exhibited increased infiltration of $CD8^+$ T cells and decreased infiltration of TAMs in HCC tissues (Extended Data Fig. 24e-h). Importantly, 38 patients received ICB therapy before hepatectomy, with 14 patients achieving a partial response (PR) and 24 patients displaying progressive disease (PD). We found that patients with low circPETH levels exhibited a significantly greater response to ICB therapies (Extended Data Fig. 24i), which preliminarily implies a crucial role for circPETH in immunotherapy for HCC in patients."

The data were also shown as below:



3. One of their major findings is that circPETH-147aa impairs anti-HCC immunity by inducing methionine and leucine deficiency in cytotoxic CD8+ T cells. However, flow cytometry analysis by co-culturing tumor cell with T cell is too rough to detect T cell anti-tumor activity because it cannot reflect specific anti-tumor response. Tumor-specific antigen, such as OVA, should be established on tumor cells, or at least analysis immune profile in TME by in-vivo HCC mouse model.

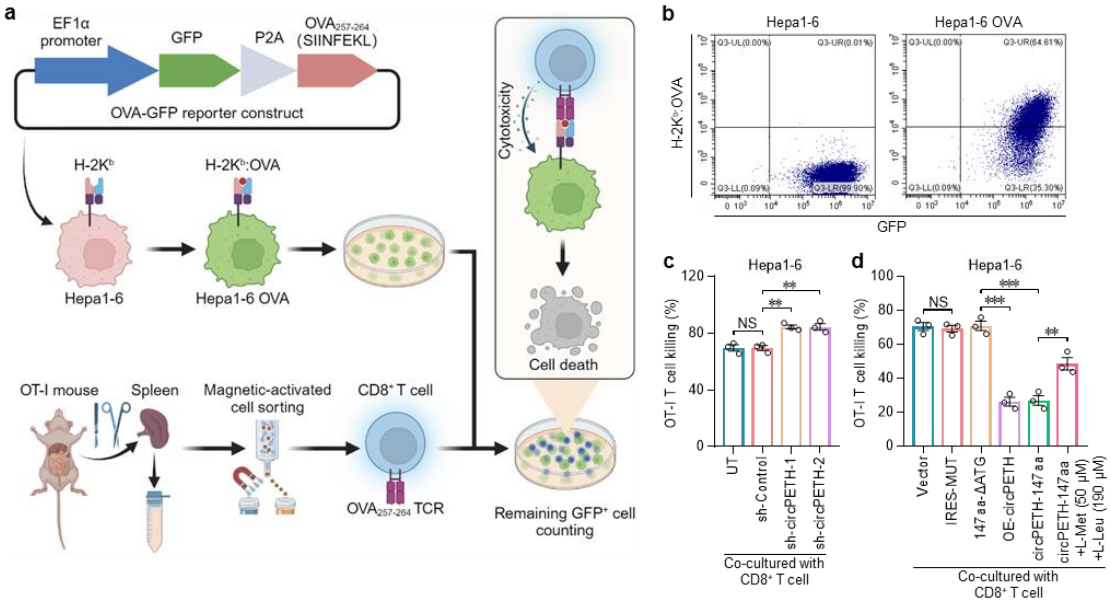
Response:

Thank you for your kind suggestion. We have added experiments to reflect specific anti-tumor response. Below is the newly added text.

Page 23: "To assess the specific antitumour response, we engineered Hepa1-6 cells, well-characterized H-2K^{b}+ mouse HCC cells, to express a truncated form of the tumour antigen ovalbumin257-264 (OVA$_{257-264}$) (Extended Data Fig. 24a). To evaluate the expression of antigen-specific pMHC-I, we used a unique mouse TCR-mimicking antibody that binds the immunogenic OVA$_{257-264}$ peptide presented by the H-2K^{b} molecule (H-2K^{b}:OVA$_{257-264}$, hereafter referred to as H-2K^{b}:OVA) to verify successful antigen presentation (Extended Data Fig. 24b). Correspondingly, OT-I T cells, a type of primary mouse CD8^{+} T cells expressing a unique T-cell receptor (TCR) that specifically recognizes H-2K^{b}:OVA, were isolated from the spleens of OT-I TCR transgenic mice and subsequently cocultured with Hepa1-6 OVA cells (Extended Data Fig. 24a); the results revealed that circPETH-deficient Hepa1-6 OVA cells were more susceptible to OT-I T-cell-mediated killing (Extended Data Fig. 24c). Conversely, Hepa1-6 OVA cells were more resistant to OT-I T-cell-mediated killing in the presence of circPETH-147aa, and this effect was reversed by methionine and leucine

supplementation (Extended Data Fig. 24d).”

The data were also shown as below:



4. As mentioned in the comment above, the authors performed in-vitro co-culture experiments to verify that Norathyriol boosts cytotoxic CD8+ T-cell function. The lack of in-vivo experiments making it hard to support this conclusion.

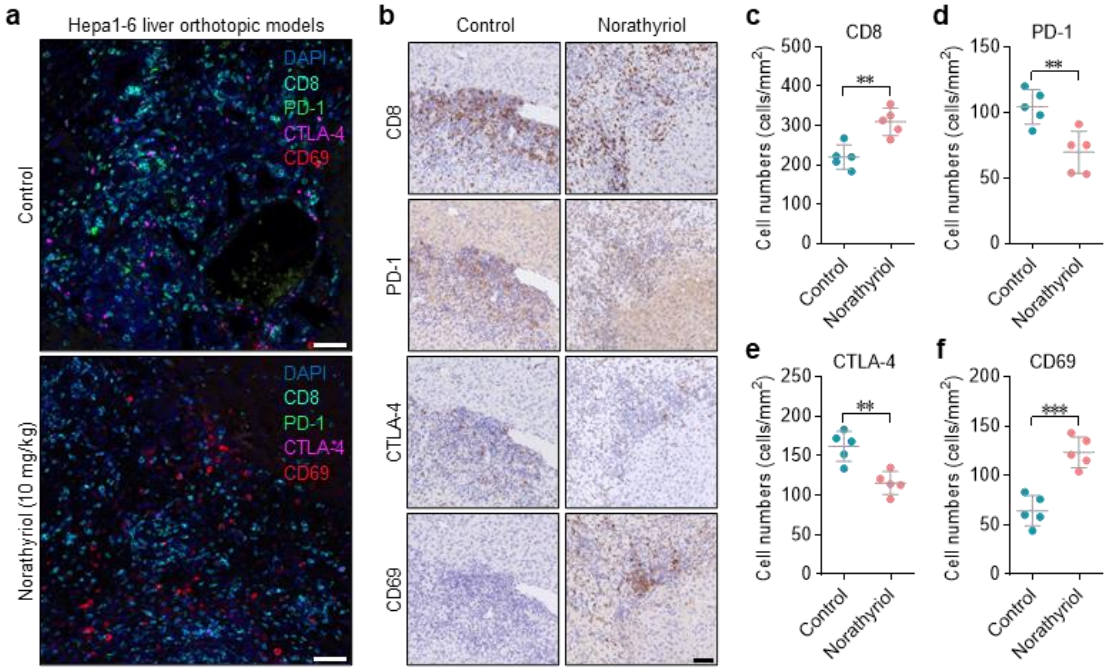
Response:

Thank you for your kind suggestion. We have detected the infiltration of $CD8^+$ T cells and the expression levels of representative markers (PD-1, CTLA-4 and CD69) in HCC tissues of Hepa1-6 liver orthotopic models using IF and IHC to determine the effects of Norathyriol on tumor-infiltrating $CD8^+$ T cells in vivo. The results showed that Norathyriol treatment was associated with significant higher infiltration of $CD8^+$ T cells, higher expression of CD69 and lower levels of PD-1 and CTLA-4 (Extended Data Fig. 27a-f), indicating that Norathyriol strengthened tumor-infiltrating $CD8^+$ T-cell function. Below is the newly added text.

Page 25, 26: “Consequently, we analysed the infiltration of $CD8^+$ T cells and the expression levels of representative markers (PD-1, CTLA-4 and CD69) in HCC tissues via IF and IHC to determine the effects of norathyriol on tumour-infiltrating $CD8^+$ T cells in vivo. The results revealed that norathyriol treatment was associated

with significantly increased infiltration of $CD8^+$ T cells, increased expression of CD69 and decreased levels of PD-1 and CTLA-4 (Extended Data Fig. 27a-f), indicating that norathyriol strengthened the function of tumour-infiltrating $CD8^+$ T cells."

The data were also shown as below:



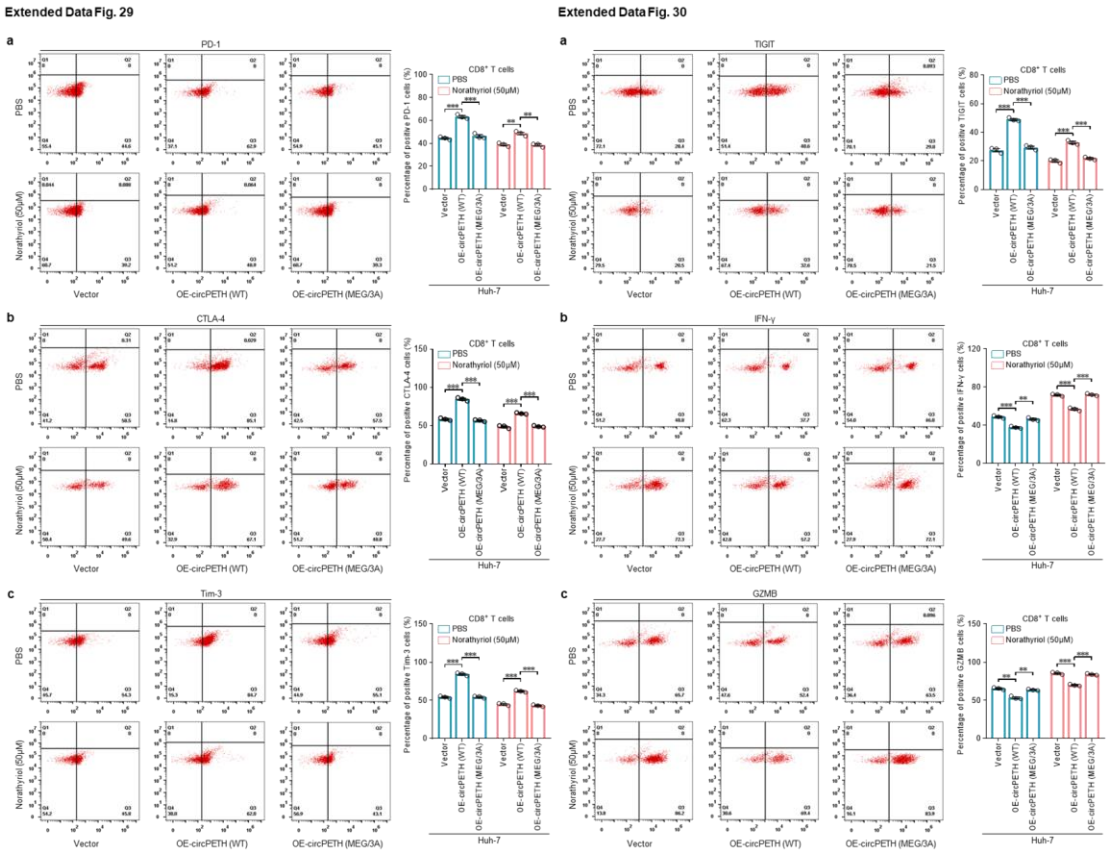
5. The authors examined the relative expression levels of several markers (such as PD-L1, CTLA4, IFN-r, and GZMB) on CD8+ T cells using RT-PCR. However, flow cytometry is a more appropriate technique for detecting specific cell populations, such as PD-L1+ CD8+ T cells, as it allows for the quantification of surface marker expression levels, which are more directly relevant to their functional roles in the tumor microenvironment (TME).

Response:

Thank you for your kind suggestion. We have detected the expression levels of PD-1, CTLA4, Tim-3, TIGIT, IFN-γ and GZMB on $CD8^+$ T cells using flow cytometry and calculated the percentages of these positive surface markers. The results showed that Norathyriol treatment led decreased levels of PD-1, CTLA-4, Tim-3 and TIGHT

(Extended Data Fig. 29a-c and Extended Data Fig. 30a) and increased levels of IFN-γ and GZMB (Extended Data Fig. 30b,c) on $CD8^+$ T cell surface, which was consistent with the data of RT-PCR.

The data were also shown as below:



Minor points:

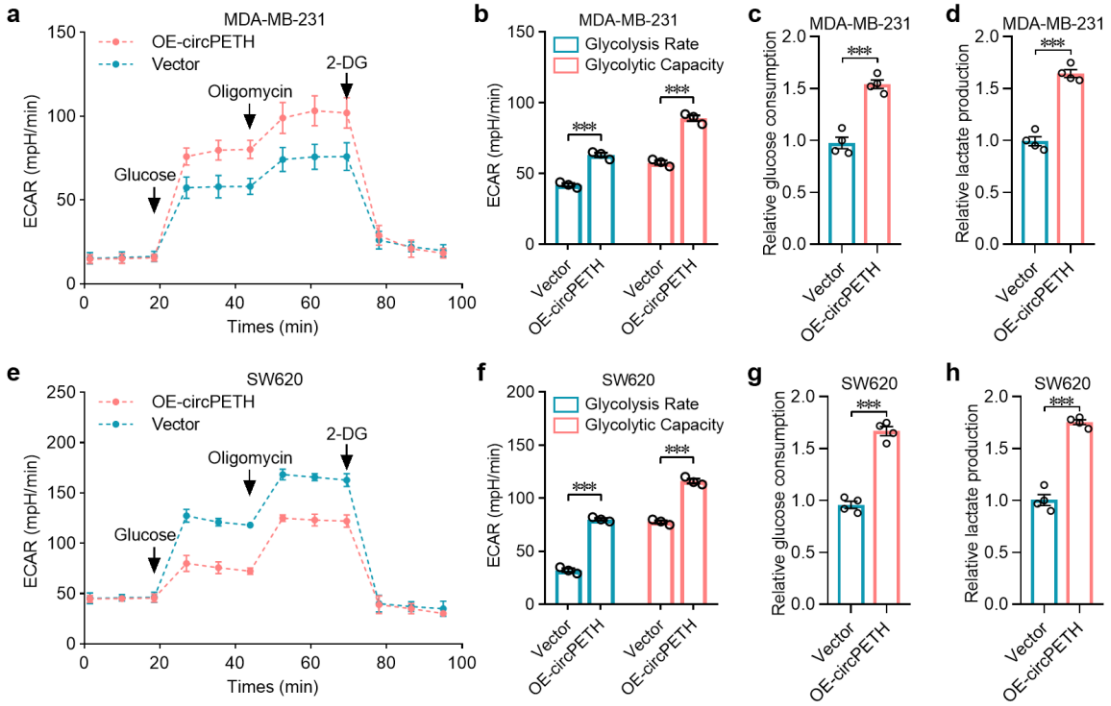
1. Are these findings specific for HCC? The authors should show data to address this to increase clinical relevance.

Response:

Thank you for your kind suggestion. We have overexpressed circPETH in human breast cancer cells and colorectal cancer cells, and determined the change on glycolysis. The results showed that upregulation of circPETH significantly promoted the glycolysis rate, glycolytic capacity, glucose consumption and lactate production of two different human cancer cell lines (MDA-MB-231 and SW620), indicating that the findings in our previous work might not be specific for HCC. However, the in vivo

experiments and the effects of Norathyriol on cancer immune should be further clarified, which was the one of our future research focuses.

The data were shown as below:



2. The gating strategies for flow cytometry analysis should be provided in the manuscript or supplementary materials.

Response:

Thank you for your kind suggestion. Representative flow cytometry data with gating strategies have been provided in "Source Data" file according to editorial requests.

3. The resolution of Extended Data Fig. 19b should be improved for better visualization and interpretation.

Response:

Thank you for your kind suggestion. That image has been provided with high resolution (Extended Data Fig. 20b).

Thank you again for helping us to revise our manuscript and improve this work.

With kind regards!

Hong Wu, MD., Prof. (on behalf of the authors)

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Point-by-point response to the reviewer comments

Dr. Reviewers

We would like to thank you for your valuable comments, which are essential for us to further improve the quality and accuracy of our manuscript. Those comments are very helpful for improving our work.

Reviewers' comments

Reviewer #1 (Remarks to the Author):

The authors have answered all of my questions. The manuscript is acceptable for publication.

Thank you for your recognition of our work.

Reviewer #2 (Remarks to the Author):

The authors' answers completely resolved my doubts. So I am satisfied with that answer. Next, I support publication.

Thank you for your recognition of our work.

Reviewer #3 (Remarks to the Author):

The authors have addressed all the questions I raised and have carefully considered each of my comments in their responses. They provided detailed clarifications where needed and made adjustments to the manuscript accordingly. I am satisfied with the revisions made and believe the manuscript is easier to understand now. Thank you for facilitating this exchange.

Thank you for your recognition of our work.

Reviewer #4 (Remarks to the Author):

Thank you for the response to my queries associated with the virtual screening. I'm

happy with the responses and make no further comment or requests of the authors

Thank you for your recognition of our work.

Reviewer #5 (Remarks to the Author):

I appreciate the revisions to the manuscript resubmitted by the authors. They have extensively revised and clarified the major questions raised by the reviewers, and have provided new data that strengthens their work.

Thank you for your recognition of our work.

Thank you again for helping us to revise our manuscript and improve this work.

With kind regards!

Hong Wu, MD., Prof. (on behalf of the authors)

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