Peer Review File

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Reply to Reviewer A

Thank you for your patient and careful comments, which have well demonstrated your unique academic insights and profound academic attainments, bringing great enlightenment and help to our scientific research ideas. We have thoroughly revised the manuscript according to your requirements, hoping to meet your requirements. Here's my point-to-point response to your questions:

Comment 1: First, the title needs to indicate that this is a bioinformatics analysis based on existing database.

Reply 1: Thank you for your suggestions. Indeed, the previous title of the paper was confusing and did not reflect the research methodology of this article, and we accept your suggestion to change the title of the article.

Changes in the text:

Identification of potential biomarkers associated with CD4+ T cell infiltration in myocardial ischemia-reperfusion injury using bioinformation analysis

Comment 2: Second, the abstract needs further revisions. The background needs to indicate the potential clinical significance of this study and what the clinical need is. The methods need to describe how the biomarkers were identified and how their sensitivity and accuracy were analyzed. The conclusion needs comments for the implications of the findings.

Reply 2: Thank you for your valuable and instructive suggestions. Our abstract does have areas for improvement and we have revised this paper in line with your suggestions.

Changes in the text:

Abstract

Background: Myocardial ischemia-reperfusion injury (MIRI) is often part of clinical events such as cardiac arrest, resuscitation, and reperfusion after coronary artery occlusion. Recently, more and more studies have shown that the immune microenvironment is an integral part of ischemia-reperfusion injury (IRI), and CD4+ T-cell infiltration plays an important role, but there are no relevant molecular targets for clinical diagnosis and treatment.

Methods: The transcriptome data and matched group information were retrieved from the Gene Expression Omnibus (GEO) database. The ImmuCellAI-mouse algorithm was used to calculate each symbol's CD4T cell infiltration score. The time period with the greatest change in the degree of $CD4^+$ T cell infiltration [ischemia-reperfusion 6 hours (IR6h)–ischemia-reperfusion 24 hours (IR24h)] was selected for the next analysis. Weighted gene co-expression network analysis (WGCNA) and differential expression analysis were performed to screen out $CD4^+$ T cell-related genes and from which the gene CLEC5A was screened for the highest correlation with CD4+ T cell infiltration. The potential regulatory mechanism of $CD4^+$ T cells in MIRI was discussed through various enrichment analysis. Finally, we analyzed the expression and molecular function (MF) of *CLEC5A* and its related genes in MIRI.

Results: A total of 406 $CD4^+$ T cell-related genes were obtained by intersecting the results of WGCNA and differential expression analysis. Functional enrichment analysis indicated that the

$CD4^+$ T cell-related genes were mainly involved in chemokine signaling pathway and cell cycle. By constructing a protein-protein interaction (PPI) network, a total of 12 hub genes were identified as candidate genes for further analysis. Through the correlation analysis between the 12 candidate genes found in the PPI network and $CD4^+$ T cell infiltration fraction, we determined the core gene *CLEC5A*. Finally, a gene interaction network was constructed to decipher the biological functions of *CLEC5A* using GeneMANIA.
Conclusions: In this study, RNA sequencing (RNA-Seq) data at different time points after reperfusion were subjected to a series of bioinformatics methods such as PPI network, WGCNA module, etc., and CLEC5A, a pivotal gene associated with $CD4^+$ T-cells, was found, which may serve as a new target for diagnosis or treatment.

Comment 3: Third, in the introduction of the main text, the authors need to review what has been known on the prognostic biomarkers associated with CD4+ T cell infiltration in myocardial ischemia-reperfusion injury, analyze what the knowledge gap is and what limitations of prior studies are, and further clarify the study hypotheses. The last paragraph should not describe what the authors did and found, please describe the clinical significance and the questions to be answered. The current study is not an observational study, so STROBE is wrong.

Reply 3: Thank you for your comments, you have provided us with constructive feedback on the writing of the paper and we have reworked the introduction of the main text as per your suggestions. In addition, we have made the cuts in the introductory section and revised the reporting checklist as you requested.
Changes in the text:
Adaptive immune responses, particularly those involving CD4+ T cells, play an important role in ischemia-reperfusion. Several studies have used CD4+ T cell-deficient mice and demonstrated a critical role for CD4+ T cells in IR injury and infarct healing. However, there is a lack of suitable biomarkers for predicting the extent of CD4+ T cell infiltration in ischemia-reperfusion. The use of biosignature analysis to solve challenging biological problems will not only help advance basic research, but may also provide new breakthroughs in biomedical applications.

Comment 4: Fourth, at the beginning of the methodology, please have a brief overview of the research procedures of this study and what the questions to be answered by each procedure. Please also describe the statistical methods for assessing the prognostic effect of the biomarkers, i.e., accuracy and specificity.

Reply 4: Thank you for your guidance and this study is mainly a bioinformatics analysis based on the mRNA sequencing results of mouse myocardial ischemia-reperfusion samples, and lacks relevant clinical studies to determine the prognosis of patients. This study focuses on the changes of ischemia-reperfusion injury and T-cell infiltration with and related genes, hoping to find markers or targets for diagnosis and treatment. However, the bioinformatics analysis does not necessarily match the actual, and subsequent biochemical experiments are needed to verify this, which is a limitation of our study.

Comment 5: Finally, please consider to review and cite several related papers: 1. Zhang Z, Zhou M, Liu H, Liu W, Chen J. Protective effects of Shen Yuan Dan on myocardial ischemia-reperfusion injury via the regulation of mitochondrial quality control. Cardiovasc Diagn Ther 2023;13(2):395-407. doi: 10.21037/cdt-23-86. 2. Zhang D, Zheng N, Fu X, Shi J, Zhang J. Dl-3-n-butylphthalide attenuates myocardial ischemia reperfusion injury by suppressing oxidative stress and regulating cardiac mitophagy via the PINK1/Parkin pathway in rats. J Thorac Dis 2022;14(5):1651-1662. doi: 10.21037/jtd-22-585.

Reply 5: Thank you for your suggestion, we have read the papers you have provided and they are indeed very valuable and instructive for this paper and our subsequent research, as well as we have cited both of them.

Reply to Reviewer B

The paper titled “Identification of potential prognostic biomarkers associated with CD4+ T cell infiltration in myocardial ischemia-reperfusion injury” is interesting. This study involved a bioinformatics analysis of RNA sequencing (RNA-Seq) data at different time points after reperfusion. Then, a series of bioinformatics methods including a PPI network and WGCNA module were used to find the hub gene related to CD4+ T cells. However, there are several minor issues that if addressed would significantly improve the manuscript.

Reply: Thank you for your patient and careful comments, which have well demonstrated your unique academic insights and profound academic attainments, bringing great enlightenment and help to our scientific research ideas. We have thoroughly revised the manuscript according to your requirements, hoping to meet your requirements. Here's my point-to-point response to your questions:

Comment 1: This study is only the result of bioinformatics analysis and requires experimental validation with a larger sample size.

Reply 1: Thank you for your suggestion. It is true that this study is only based on bioinformatics analysis and lacks a lot of experimental verification, which is also a limitation of our study. This is also one of the limitations of our study. However, due to the existing experimental conditions, it is difficult for us to carry out a large number of biochemical experiments for in-depth research. Our research is dedicated to solving a challenging biological problem using biosignature analysis, which not only helps to advance basic research, but also may provide new breakthroughs for biomedical applications. In the era of big data, biosignature analysis has become an indispensable tool for understanding genomic, proteomic and transcriptomic data. Such analysis helps to shed light on important issues such as disease mechanisms, biodiversity and evolution.

Comment 2: The abstract is not sufficient and needs further modification. The research background did not indicate the clinical needs of the research focus.

Reply 2: Thank you for your valuable and instructive suggestions. Our abstract does have areas for improvement and we have revised this paper in line with your suggestions.

Changes in the text:

Abstract

Background: Myocardial ischemia-reperfusion injury (MIRI) is often part of clinical events such as cardiac arrest, resuscitation, and reperfusion after coronary artery occlusion. Recently, more and more studies have shown that the immune microenvironment is an integral part of ischemia-reperfusion injury (IRI), and CD4+ T-cell infiltration plays an important role, but there are no relevant molecular targets for clinical diagnosis and treatment.

Methods: The transcriptome data and matched group information were retrieved from the Gene Expression Omnibus (GEO) database. The ImmuCellAI-mouse algorithm was used to calculate each symbol's CD4T cell infiltration score. The time period with the greatest change in the degree of CD4+ T cell infiltration [ischemia-reperfusion 6 hours (IR6h)–ischemia-reperfusion 24 hours (IR24h)] was selected for the next analysis. Weighted gene co-expression network analysis (WGCNA) and differential expression analysis were performed to screen out CD4+ T cell-related genes and from which the gene CLEC5A was screened for the highest correlation with CD4+ T cell infiltration. The potential regulatory mechanism of CD4+ T cells in MIRI was discussed through various enrichment analysis. Finally, we analyzed the expression and molecular function (MF) of CLEC5A and its related genes in MIRI.

Results: A total of 406 CD4+ T cell-related genes were obtained by intersecting the results of WGCNA and differential expression analysis. Functional enrichment analysis indicated that the CD4+ T cell-related genes were mainly involved in chemokine signaling pathway and cell cycle. By constructing a protein-protein interaction (PPI) network, a total of 12 hub genes were identified as candidate genes for further analysis. Through the correlation analysis between the 12 candidate genes found in the PPI network and CD4+ T cell infiltration fraction, we determined the core gene CLEC5A. Finally, a gene interaction network was constructed to decipher the biological functions of CLEC5A using GeneMANIA.

Conclusions: In this study, RNA sequencing (RNA-Seq) data at different time points after reperfusion were subjected to a series of bioinformatics methods such as PPI network, WGCNA module, etc., and CLEC5A, a pivotal gene associated with CD4+ T-cells, was found, which may serve as a new target for diagnosis or treatment.

Comment 3: Figure 2C is not clear enough. It is recommended to provide clearer figure again.

Reply 3: Thank you for your comments, we have re-provided the figure with better clarity.

Comment 4: The pathophysiological mechanism and research progress of myocardial ischemia-reperfusion injury should be added to the discussion.

Reply 4: Thank you very much for your advice. We have added a section on pathophysiologic mechanisms and research advances in myocardial ischemia-reperfusion injury in the discussion section.

Changes in the text:

The pathophysiological mechanisms of myocardial ischemia-reperfusion injury are complex, but progress is being made in this area of research to explore more effective preventive and therapeutic approaches to achieve the goal of mitigating the impact of myocardial reperfusion injury on heart health. Existing research findings are mainly related to inflammatory responses,

oxidative stress, and calcium overload. Researchers are also currently exploring new approaches such as the use of antioxidants, inhibition of inflammatory responses, stem cell therapy or gene therapy to protect cardiomyocytes. This will hopefully improve the quality of life and prognosis of heart disease patients.

Comment 5: It is recommended to add in vivo and in vitro experiments to study the biological function of CLEC5A.

Reply 5: Thank you for your suggestion. It is true that this study is only based on bioinformatics analysis and lacks a lot of experimental verification, which is also a limitation of our study. This is also one of the limitations of our study. We clearly recognize that experimental studies can better reveal the molecular mechanisms of disease development. However, due to the existing experimental conditions, it is difficult for us to carry out a large number of biochemical experiments for in-depth research. Our research is dedicated to solving a challenging biological problem using biosignature analysis, which not only helps to advance basic research, but also may provide new breakthroughs for biomedical applications. In the era of big data, biosignature analysis has become an indispensable tool for understanding genomic, proteomic and transcriptomic data. Such analysis helps to shed light on important issues such as disease mechanisms, biodiversity and evolution.

Comment 6: The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as “Protective effects of Shen Yuan Dan on myocardial ischemia-reperfusion injury via the regulation of mitochondrial quality control, Cardiovasc Diagn Ther, PMID: 37583687”. It is recommended to quote this article.

Reply 6: Thank you for your suggestion, we have read the paper you provided and it is indeed very valuable and instructive for this paper and our subsequent research, which we also cite.

Comment 7: How to judge the prognostic characteristics of myocardial ischemia-reperfusion injury based on the results of this study? How to provide candidate targets for the treatment of myocardial ischemia-reperfusion injury? It is recommended to include relevant descriptions in the discussion.

Reply 7: This study is mainly a bioinformatics analysis based on the mRNA sequencing results of mouse myocardial ischemia-reperfusion samples, and lacks relevant clinical studies to determine the prognosis of patients. This study focuses on the changes of ischemia-reperfusion injury and T-cell infiltration with and related genes, hoping to find markers or targets for diagnosis and treatment. However, the bioinformatics analysis does not necessarily match the actual, and subsequent biochemical experiments are needed to verify this, which will be our future work.