Peer Review File

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Reply to Reviewer A

We sincerely thank the reviewer for comments. We have carefully modified the manuscript in response to the reviewer's constructive and insightful comments.

Comment 1:

Over the past decades, lot of genes have been described in relation to ischemia reperfusion injury (IRI), such as pro-inflammatory genes as interleukin-1 beta (IL-1β), IL-6, P-selectin and monocyte chemotactic protein 1 (MCP-1), or apoptosis-related gene expression levels (BAX, BCL2), as well as other potential biomarkers for renal IRI, such as SPRR2F, SPRR1A, MMP-10, Malat1, and miR-139-5p. This study discovered a novel biomarker of DGF caused by IRI, but this research does not show a truly "translational" approach, so I think authors could explain better the relevance of this novel target for the prevention and treatment of DGF after kidney transplantation.

Reply 1:

As the reviewer mentioned, many potential biomarkers for renal IRI have been found in recent years. The novelty of the present study is that we found CLCF1 is a molecule linking IRI to DGF through our overlap analysis. This study can provide clues for the development of novel biomarkers of IRI-induced DGF.

According to the reviewer's suggestion, we have revised the previous issue "this novel target for the prevention and treatment of DGF after kidney transplantation" in the manuscript.

Changes in the text:

We have modified our text as advised (see Page 3, line 10-23 "IRI contributes to DGF along…."; see Page 12-13, line 21-2 "Medical products that limit….").

Comment 2:

I have one main methodological question: The authors stated that "A murine model of renal IRI was used to screen the differentially expressed genes using high-throughput assays. The differentially expressed genes was overlapped with a published DGF database, CLCF1 was an unreported most up-regulated genes in kidney IRI". However, the design of this study is not comparing DGF-grafts and non-DGF grafts. Is always IRI equivalent to DGF outcome?

Reply 2:

IRI does not correlate directly with DGF outcome but is closely related to DGF outcome.

IRI is recognized as one of the most important risk factors of DGF (1, KV Melih, B Boynuegri, C Mustafa, *et al*. "Incidence, risk factors, and outcomes of delayed graft function in deceased donor kidney transplantation". *Transplantation Proceedings.* May 2019; 1096-1100. 2, M Ounissi, M Cherif, TB Abdallah, *et al*. "Risk factors and consequences of delayed graft function". *Saudi Journal of Kidney disease and transplantation.* 2013; 243-246.). However, there is currently no clear "bridge" molecule linking IRI to DGF. Therefore, the purpose of this research was to identify novel "bridge" molecules. By using the murine model of renal IRI and the overlapping data from the database of DGF patients, we were able to screen for the potential "bridge" molecules of IRI-induced DGF. Although IRI is closely related to DGF, the present research mainly focused on the expression and regulation of CLCF1 in renal IRI. Therefore, we compared CLCF1 in renal IRI mouse models and the DGF database.

Changes in the text:

We have modified our text as advised (see Page 3, line 10-23 "IRI contributes to DGF along…."; Page 10, line 15-23 "CLCF1 has been identified…".)

Comment 3:

The results are shown in figures and diagrams, but it could be more illustrative if a table is provided, summarizing the n per group, the mean value of level of CLCF1, the other genes obtained.

Reply 3: We thank the reviewer for the suggestion. We have provided the required data in Supplementary Table S2.

Changes in the text:

We have added the Supplementary Table S2.

Comment 4:

On the other hand, the biological plausibility proposed for this finding is as follows: the generation of CLCF1 is controlled by FOXO3, which is a known transcription factor associated with IL-6(10). Nevertheless, the implication IL-6 in IRI is well-established in the medical literature.

Reply 4:

The IL-6 family of cytokines consists of IL-6, IL-11, IL-27, IL-31, oncostatin M (OSM), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1), and cardiotrophin-like cytokine factor 1 (CLCF1), among others. Although the association between FOXO3 and IL-6 has been well established, the expression of other members in the IL-6 family is controlled differently from IL-6, which has been reported previously (1, Yanagisawa M, Nakashima K, Arakawa H, *et al*. "Astrocyte differentiation of fetal neuroepithelial cells by interleukin-11 via activation of a common cytokine signal transducer, gp130, and a transcription factor, STAT3". *Journal of Neurochemistry.* January 18, 2002; 1498-1504. 2, Jutta HH, Harald

S, Sebastian L, *et al*. “Dendritic cells activated by IFN-γ/STAT1 express IL-31 receptor and release proinflammatory mediators upon IL-31 treatment”. *The Journal of Immunology.* June 1, 2012, 188 (11); 5319-5326. 3, P Gao, M Yuan, X Ma, *et al*. “Transcription factor Fli-1 positively regulates lipopolysaccharide-induced interleukin-27 production in macrophages”. *Molecular Immunology.* March 2016; 184-191). The main purpose of this study was to investigate whether CLCF1, rather than IL-6, could be regulated by FOXO3, so we analyzed CLCF1 expression in FOXO3 knockout mice.

On the other hand, although IL-6 has been well-established in IRI, IL-6 itself was not included in the 107 differentially expressed genes from our overlap analysis, indicating that IL-6 might be not a “bridge” molecule linking IRI to DGF. Therefore, from this aspect, we speculate that the action of IL-6 is different from CLCF1 in IRI-induced DGF.

Changes in the text:

We have modified our text as advised (see Page 4, line 9-15 “However, the regulation of CLCF1 expression…”; see Page12, line 2-7 “A recent study reported that…”)

Reply to Reviewer B

We sincerely thank the reviewer for comments. We have carefully modified the manuscript in response to the reviewer's constructive and insightful comments.

Comment 1: a protocol number of ethical committee must be informed.

Reply 1:

Thank you for the comment. We have already provided the protocol number of the ethics committee in the footnote. (Please see Page 13, Line 295-300, Ethical statements. "The animal experiments in this study were approved by the Beijing Friendship Hospital Animal Care and Use Committee (institutional approval No: 18-2022).")

Changes in the text:

N/A

Comment 2: an extensive English review should be carried out. At some points it is difficult to understand the text

Reply 2: We have had the manuscript revised by AME Editing Service.

Changes in the text:

We have modified our text as advised.

Comment 3: The author comments about transplantation, but it was not performed in this study. Thus, the authors must change the foucus of the manuscript

Reply 3:

We have changed the focus of the manuscript to renal IRI, as suggested by this reviewer. However, because CLCF1 was identified in the overlap analysis between the kidney transplantation DGF and renal IRI databases, we speculated that CLCF1 might also be associated with kidney transplantation DGF. Therefore, this aspect was also discussed in the manuscript.

Changes in the text:

We have modified our text as advised (see Page 3, line 10-23 "IRI contributes to DGF along…."; see Page 10, line 2-13 "IRI, an inevitable event during…"; see Page 10, line 15-23 "CLCF1 has been identified as an unreported....")

Comment 4: The microarray analysis must be clarify. How about de n, score, etc..

Reply 4: According to the reviewer's suggestion, we have revised the article.

Changes in the text:

We have modified our text as advised (see Page 8, line 5-19 "To explore the differentially expressed genes…")

Comment 5: Did the authors observe any alterations in renal injury markers not only creatinine? Why the time point of 24h was choosen?

Reply 5:

We did not examine other injury markers except for creatinine, because serum creatinine is the most widely used marker in the clinical evaluation of kidney function. The IRI mouse model is a widely used acute renal injury model, and a 24-hour period is applied for the construction of the model in many published papers (1, AJ Dare, EA Bolton, GJ Pettigrew, *et al*. "Protection against renal ischemia–reperfusion injury in vivo by the mitochondria targeted antioxidant Mito Q". *Redox Biology.* August 2015; 163-168. 2, C Shingu, H Koga, S Hagiwara, *et al*. "Hydrogen-rich saline solution attenuates renal ischemia–reperfusion injury". *Journal of Anesthesia*. 18 May, 2010; 569–574).

Changes in the text:

N/A

Comment 6: The FOXO3 knockout mice (sham group) showed an increase on CLCF1 levels when compared to WT. Why?

Reply 6:

We do not know the mechanism through which CLCF1 was increased in FOXO3 knockout mice. We speculate that CLCF1 may be regulated by FOXO3 either directly or indirectly. As FOXO3 is a known transcription factor and inhibits the expression of IL-6, the fact that CLCF1 levels increased in FOXO3 knockout mice supports the hypothesis that FOXO3 may negatively regulate CLCF1 expression.

Changes in the text:

N/A

Comment 7: What is the mortality rate of this model in knockout animals?

Reply 7: There were no deaths in FOXO3 knockout animals in our experiment, and we observed no phenotypic or developmental differences between FOXO3 knockout and wild type mice.

Changes in the text:

N/A

Comment 8: All the figure legends must be improved, including statistical analysis

Reply 8: We have revised the corresponding places according to the author's advice.

Changes in the text:

We have modified our figure legends as advised.

Comment 9: Did the authors evaluated the inflammatory status in all groups?

Reply 9:

We did not evaluate the inflammatory status in all groups. Instead, we evaluated the inflammatory status in the 107 overlapping genes. By using GO enrichment, we found that genes related to positive regulation of NF-kappaB transcription factor activity, positive regulation of inflammatory response, neutrophil migration, neutrophil chemotaxis, leukocyte cell-cell adhesion, granulocyte chemotaxis, cytokine receptor activity, and acute inflammatory response contributed to both IRI and DGF kidney transplantation. Please see Figure 1C and Supplementary Table S1.

Changes in the text:

N/A

Comment 10: Teh authors must include the hytological analysis of KO groups

Reply 10:

We speculate that the reviewer's question might refer to the histological analysis of knockout groups. Unfortunately, we were not able to provide these data because all the tissues had been frozen at - 80°C, and we were not able to perform histological analysis.

Changes in the text:

N/A