Author’s response to reviews

Title: Bioinformatics analyses on the immune status of renal transplant patients, a systemic research of renal transplantation

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Author’s response to reviews:

Reviewer #1:

In the article entitled "Bioinformatics analyses on the immune status of renal transplant patients, systemic research of renal transplantation", Meng M and his colleagues systematically investigated differences of gene expression among different groups of renal transplant recipients by using public data from GEO. This study is well conducted though the results show limited novelty in gene expression of transplant patients.

In chronic rejection group compared with stable recipients, expression differences were enriched in allograft rejection module; in acute rejection patients compared with stable recipients, genes with significant differences were enriched in modules of allograft rejection and immune deficiency. As that was expected, MHC molecules and immunoglobulins were increased in both acute and chronic rejection while expressions of LILRB and MAP4K1 were increased in acute rejection patients but not in stable recipients.
Kidney transplantation is the most effective treatment for end-stage renal disease. This manuscript provided useful information for further exploring the molecular mechanism against transplant rejection.

Comments:

1. In the 'Background' section, previous discoveries are not fully addressed, particularly the discoveries from the published GEO data sets.

   We have added some previous findings from literature in the introduction (Page4, paragraph 1).

2. As with the abbreviations, spell out the full name upon first mention in the text and then provide the abbreviation. The full name of abbreviation 'SEM' is not found in the text. Please also check the text for the other abbreviations.

   SEM stands for standard errors of means. As suggested, we have checked through and corrected in the manuscript.

3. In general, symbols for genes are italicized, whereas symbols for proteins are not italicized. Please check the symbols in the text carefully.

   As suggested, we have italicized genes in the manuscript.

Reviewer #2:

The author investigated immune status changes in renal transplant recipients and reported a number of differentially expressed genes (DEGs) but comparing chronic, acute rejection group with stable recipients and healthy samples. Their analysis was based on fifteen datasets from Gene Expression Omnibus (GEO).

The authors need to address the following questions.
The major questions are:

1. The study reports several DEGs in the renal biopsy of transplant recipients based on microarray datasets with limited sample size in the GEO database. The authors should use clinical samples to validate the discovery of DEGs.

Thank you very much for the constructive suggestions. We have been planned to validate clinical samples since day 1. There are some reasons that we could not perform the validation in the present study and we sincerely hope to get understanding from reviewers and editors. 1) We do have some biopsy samples for clinical diagnosis. After regular HE staining as well as several IHC and IF examinations, there were no much samples left. 2) Due to the preciousness of transplant graft, transplant recipients and their family, both stable and rejection subjects, reluctant to perform regular biopsies or get one more sample during the biopsy procedure. 3) Regarding the above issues, the ethical committee in the university hospital has not fully approved our request for using graft biopsy samples in research.

We hope that we could confirm our findings in transplant patients soon.

2. The authors mentioned there are several confounding factors such as innate immunity, inflammation and microcirculation remodeling, which complicates the understanding of renal allograft rejection. Is the contribution of these factors properly evaluated in the 15 GEO studies after applying the simplified filtering criteria?

As suggested, we have added finding from literature in the introduction.

In the present study, DEGs in the comparison of acute rejection patients with stable subjects were enriched in allograft rejection, chemokine signalling pathway module, primary immunodeficiency, NF-κB signalling, TLR signalling, as well as PPAR signalling module. The DEGs in the comparison of stable recipients and healthy subjects were enriched in chemokine signalling, prion diseases, TLR signalling, endometrial cancer, long-term potentiation, as well as shigellosis modules. We believe these nonspecific immune response-related signalling changes are due to inflammation, microenvironments, as well as innate immune response. The pattern changes in the progress of transplant status indicate that the balance between immune defence and attack play a critical role in transplant immunology, and inflammation-related-signalling pathways play a role in undermining the immune balance.
3. The description of the datasets is limited in the methods. Since there is no validation, a natural question about the design of current study is, how much will the result of DEGs change with the size of available dataset? Note that the authors arbitrarily removed GSE9493 from seven datasets just because of the low number of DEGs found. This raised the concern about ascertainment bias in the choice of the datasets in GEO which can affect the results. Also, how to evaluate the candidate DEGs in the 5 out of 6 datasets when comparing acute rejection vs stable recipients?

We did background correction and normalization to each set individually. We have addressed it in the method. As suggested, we have attached file (S table 1-3), in which candidate DEGs in progress changes of immune status were quantified, together with the corresponding P-value. We shall include it in the manuscript if reviewers and editors request.

Regarding the dataset GSE9493, we analyzed the same data for chronic rejection vs stable recipients (2657 DEGs), acute rejection vs stable recipients (6 DEGs), and stable recipients vs healthy subjects (3164 DEGs). There were no overlapping genes when we pooled all the 7 datasets together, compared acute rejection with stable recipients. Currently, we do not have a straight answer to it. As commented, DEGs in the 5 out of 6 datasets are also interesting and deserve further investigation. Therefore we proceeded our results with DEGs in the 6 of 7 datasets.

We share the same concern with reviewer 2 that samples size affects the results. Thus, we combined 15 available online data sets and conducted this systemic study. We have been planning to validate the genes since the beginning of the project. Currently, we are not able to do so (We have addressed it in answer 1). We sincerely hope to get understanding from reviewers and editors.

4. The authors did not compare their DEGs with existing finding in the literature, which is very important to highlight the novelty of this study and may also serve as a control set to evaluate the quality of analysis in the present study.

As suggested, we have added some comparison from literature in the discussion (Page 13, paragraph 1 and page 14 paragraph 2).

5. Based on the DEGs discovery in the current study, what is new in our understanding of the mechanism or regulators in the process of allograft rejections?
The online database provides substantial information. As shown in Figure 1, there are more than 100 data sets concerning renal transplant subjects. Due to different entry points and perspectives, various therapeutic regimens, and complicated outcomes, there was no consistent conclusion on the immune status evaluation, thus lacking effective treatments. We share the same concern with reviewer 2 that samples size affects the results. It prompts us to combine 15 available online data sets and conduct this systemic study. The present study focused on changes in immune status in transplant recipients. The novelty of study includes 1) Increased expressions of LILRB and MAP4K1 are potential checkpoints for the occurrence of acute rejection. 2) Gene profiles of peripheral blood lymphocytes are not in line with those of renal biopsies.

6. Most of the DEGs analysis suffer from small sample size in the comparison when stratified down to each case and data set (Table 1 and 2). The statistical significance of each candidate DEG is a big concern and needs to be quantified in the text (together with the sample size explicitly mentioned). Specifically,

As suggested, we have attached the results in the supplementary material (S table 1-3). We shall include it in the manuscript if reviewers and editors request.

7. Page 6, Line 17 (IGHM, IGHV4-31 and IGHG1 were upregulated in chronic rejection patients but not in stable recipients) - Figure 5B, IGHM and IGHV4-31 in stable are also upregulated on average (there are only 2 samples in the stable group vs 3 in the chronic rejection group, what is the p-value of the difference?).

In the comparison between stable (2 datasets: GSE9493 and GSE1563) and chronic rejection (3 datasets: GSE9493, GSE36059, GSE98320), there were 32 stable recipient’s vs 23 healthy subjects and 416 chronic rejection vs 1076 stable recipients. As suggested, we have attached the results in the supplementary material (S table 1). We shall include it in the manuscript if reviewers and editors request.
8. Page 6, Line 36-39 (Expressions of MAP4K1, LILRB2 and IGHG1 were increased in acute rejection patients but not in stable transplant recipients) - Figure 5C, from the plot, again there are only two samples of the stable patients in comparison with acute group for gene MAP4k1 and LILRB2, how significant is the fact that the expression in MAP4K1, LILRB2 in stable transplant recipients are not increased?

As suggested, we have attached the results in the supplementary material (S table 2). We shall include it in the manuscript if reviewers and editors request.

9. Page 6 Lin 47 (There were sixteen upregulated genes found in both acute and chronic rejection comparison) - Figure 5D, most of the expression fold change is within 3-fold, given the small sample size, what is the contribution from the statistical noise?

We corrected and normalized each dataset individually. The statistically significantly upregulated and downregulated genes were defined when absolute log2 FC was higher than 0.5 (1.414-fold change) and an adjusted p-value was less than 0.05. By using the Benjamini-Hochberg false discovery rate correction, the p-value was adjusted to correct multiple hypotheses. In the comparison between acute and chronic rejection, there was 6 acute data sets (GSE1563, GSE50058, GSE25902, GSE36095, GSE98320, and GSE106675) and 3 chronic data sets (GSE9493, GSE36059, GSE98320). There were 209 acute rejection vs 1245 stable recipients, and 416 chronic rejection vs 1076 stable recipients in the comparisons. As suggested, we have attached the results in the supplementary material (S table 3). We shall include it in the manuscript if reviewers and editors request.

10. Page 7, Line 12 (Of note, expressions of ... were significantly higher in the acute rejection group than those in the chronic one) - Can the authors qualify the significance of this comparison (it is not straightforward to estimate from Table 1)?

As suggested, we have attached the results in the supplementary material (S table 1-3). We shall include it in the manuscript if reviewers and editors request.
11. Page 7, Line 26: There were no DEGs overlapped in combined comparisons. - This is unexpected. While the authors provided three interpretations in the discussion, it may be helpful to evaluate the ascertainment bias in the GEO datasets and the ethnicity difference in the samples.

We were surprised at the result too. As suggested, we have checked through all the data. All 15 studies were carried out in North American or Europe. Most participants in the studies were Caucasian, while a few subjects were African-American, Asian or American Indian. There were no noticeable ethnicity differences in the present study. We made some assumptions in the manuscript since we do not have a direct answer to this.

Methods:

12. Page 15, Line 3: both upregulated and downregulated were defined when log FC was higher than 0.5 after correction with a false discovery rate - What is the false discovery rate and why such a low logFC cutoff was chosen? Since there is no validation in this study, how do the authors access the false positives in the analysis?

We have corrected it in the manuscript (Page 6, paragraph 1). The statistically significantly upregulated and downregulated genes were defined when absolute log2 FC was higher than 0.5 (1.414-fold change) and an adjusted p-value was less than 0.05. The adjusted p-value was used to correct multiple hypotheses by using the Benjamini-Hochberg false discovery rate correction

We realized that there were huge differences in the data analysis, such as absolute or adjusted p-value (with or without false discovery rate), absolute log2 FC higher than 0.5 or 1. After standardizing all the recruited data, we found that there were much fewer overlapping genes as expected. It is the main reason we lowered the cutoff value in the present study. We understood the importance of validation in the present study. As mentioned in answer 1, there are some reasons that we could not perform the validation in the present study, and we sincerely hope to get understanding from reviewers and editors.

Minor questions about the format:

13. - Figure 2C, 3C, 4C are not legible
We have corrected it in the manuscript


We have corrected it in the manuscript