Response to reviewers 1 (in detailed)

Dear reviewers:

Thanks a lot for you to review our manuscript and raised many valuable comments towards our paper. We have made some corrections and promoted our manuscript according to these comments, which are shown as follows:

Reviewer #1:

The authors genotyped 300 SNPs of 69 renal allograft recipients taking sirolimus. They found that only CYP3A4 (Ch7: 99361466 C> T, rs2242480) was significantly associated with trough sirolimus levels (corrected for the doses).

1. **There are 3 CYP3A4 SNPs mentioned in this study: Ch7: 99361466 C> T, rs2242480-CC, -AC, -AA groups, and CYP3A4*1G rs2242480, G>A. Are they at the same site? Please clarify.**
2. **Abstract conclusion: 'mutation' should be polymorphism.**

Author response

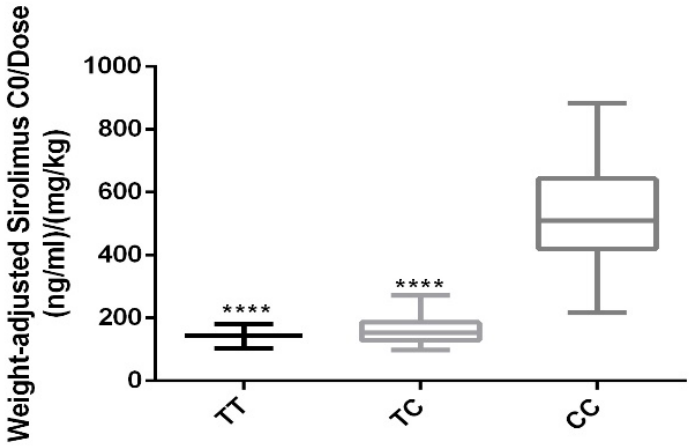
1. These SNPs mentioned in this study were the same site. In fact, the rs2242480 was located in the 99361466 of chromosome 7 with the mutation from C to T, which was also known as CYP3A4*1G. We feel so sorry for our mistakes in the subgroup analysis of Figure 2 and three groups of rs2242480 should be corrected to be –TT, -TC and –CC groups in Figure 2 and the section of "Results".

Revision

Abstract section : The mean trough SIR concentration of patients in the CYP3A4 rs2242480-CC group was more significant compared to that of the CYP3A4 rs2242480-TC and TT group, respectively 533.3; 157.4 and 142.5 (ng/ml)/mg/kg, P<0.0001.

Result section : In the total of 69 recipients, CYP3A4 rs2242480 had greater proportion than of the CC genotype than TC and TT genotypes (56.9% vs 37.3% vs 3.4%). Fig.2 shows the genotype polymorphisms that influenced the weight-adjusted SIR C0/dose. In the homozygote dominant variant CC, the mean C0 of the CYP3A4 rs2242480 was significantly higher than that for the heterozygote TC and homozygote recessive TT (P<0.0001) (533.3, 157.4, 142.5 (ng/ml)/mg/kg, respectively). The difference in the mean ± SD value between weight-adjusted SIR C0/dose of the CC and TC group CYP3A4 rs2242480 was 375.9±35.27 (ng/ml)/mg/kg) (P<0.0001). The most significant change in SIR C0 was for homozygote dominant CC, which was increased more than 3.4-fold compared with heterozygote TC and homozygote recessive TT.

Figure 2



2. We have changed the word of " mutation" with the "polymorphism" in the abstract conclusion.

Revision in conclusion section :

Conclusions: These findings indicated the significant association of polymorphism in the CYP3A4 (Ch7: 99361466 C> T, rs2242480) with SIR trough concentration after 1-year administration in patients who have undergone kidney transplantation.

We sincerely hope that these revisions are adequate, and that our revised manuscript is now acceptable for publication in "*Current Drug Metabolism*" Journal.

Best regards!

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Rebuttal Response Letter

Dear editors and reviewers:

Thanks a lot for you to review our manuscript and raised many valuable comments towards our paper. We have made some corrections and promoted our manuscript according to these comments, which are shown as follows:

Reviewer:

The manuscript entitled " A genetic polymorphism of CYP3A4 rs2242480 associated with sirolimus trough concentrations among adult renal transplant recipients" has merit, may be accepted after fulfilling the following minor concerns:

1. **My question was:**

   Also, In general, the level of significance should be corrected for multiple testing. The authors should clarify this point.

   The response was:

   In our study, we performed logistic regression analysis adjusted by significant confounding factors, limited in one statistical model, to explore the significant SNPs associated with the sirolimus concentrations in renal transplant recipients, which should be considered as one testing instead of multiple testing. Thus, we felt so appreciated for your considerations here and there was no need of the correction for multiple testing among the statistical analysis.

   My response:

   As Balding definition (2006): if n SNPs are tested and the tests are approximately independent, the appropriate per SNP significance level α' should satisfy $\alpha = 1 - (1 - \alpha')^n$, which leads to the Bonferroni correction $\alpha' \approx \alpha / n$.

   So, the significance level should be divided by the number of tested SNPs (300) even if one statistical model was used.

   For more details: Balding DJ. A tutorial on statistical methods for population association studies. Nat Rev Genet. 2006;7:781–791.

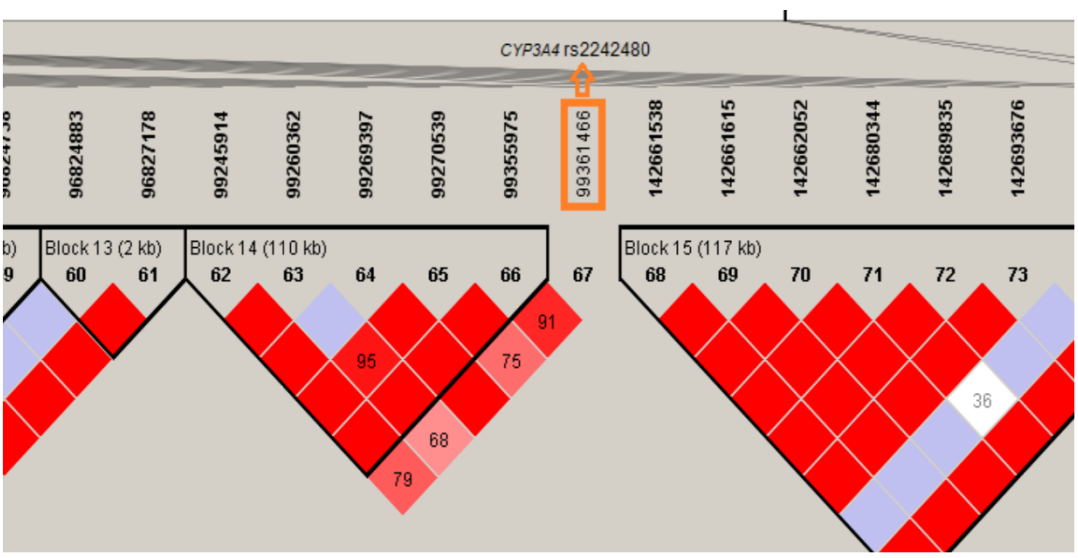
Author's response :

In our statistical analysis, we do not apply a bonferroni correction. These test is too conservative and may fail to catch some significant SNP findings. Also, some simulations show its bad fit to the real significance. Asided from that, the bonferroni designed a method of correcting for the increased error rates in hypothesis testing that had multiple comparisons. The bonferroni correction is appropriate when a single false positive in a set of test would be a problem. It is mainly useful when there are a fairly small number of multiple comparisons and looking for one or two that might be significant. Numerous GWAS and meta-analyses using 2.5M estimated allelic dosages have been reported recently and no study used the bonferroni correction, $0.05/2{,}5M = 2x10^{-8}$, as the significance level. Due to linkage disequilibrium among GWAS markers, it is generally untrue to assume that each association test on a GWAS data set is independent. Thus, applying Bonferroni correction often gives us the most conservative p-value threshold – for a typical GWAS using 300 SNPs, statistical significance of a SNP association would be set as a low as $1.6e10^{-4}$. This is usually far too stringent and result in an enormous loss of power, producing many false negatives or Type II errors (failing to declare a test significant when the null is false). It is also known that GWASs suffer from low power when the object of our study are rare novel variants of rs2242480, rare diseases of renal transplantation patient settting of variant with small effects.

2. **CYP3A4 rs2242480 should be highlighted in Supplemental Figure 1.**

Author's response :

We have highlighted the CYP3A4 rs2242480 in Supplemental Figure 1.



3. **Please give the precise link for data instead of**

   https://www.ncbi.nlm.nih.gov/sra database (SRP133091).

   Author's response :

   We have revised the precised link for our availability data :

   Genetic expression files are posted on the Sequence Read Archieve (SRA; url: https://www.ncbi.nlm.nih.gov/sra database (Accession: PRJNA432695, ID:432695).

4. **References formatting was not followed.**

   Author's response :

   We have reformatted and customized the references with ACS styles only [in square bracket] in the text and listed in the same numerical order in the reference section.

We sincerely hope that our revised manuscript could be suitable for publication in your journal.

Best regards!

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Cover Letter

March 25, 2020

Editors-in-Chief
Current Drug Metabolism
University of Houston College of Pharmacy
Houston, TX
USA

Dear Ming Hu

We hereby submit our revised manuscript entitled “A genetic polymorphism of *CYP3A4* rs2242480 associated with sirolimus trough concentrations among adult renal transplant recipients” to be considered for publication in the “Current Drug Metabolism”. Please be informed that this is a revised submission of our manuscript (BMS-CDM-2020-33). We are thankful for your kind encouragement regarding to our manuscript. Herewith we are sending our revised manuscript in accordance with the comments given by the editor that was :

1. Editor comment : “Put concentrations they found in the abstract”.
   Author comment : “We have put the concentrations that we found in the abstract”
2. Editor comment : “Chinese is not a race, and if they want to use race, use Asian. If not, just remove the word”
   Author comment : We don’t use Chinese as a race. We have removed the word from the manuscript.

Lastly, we would like to thank you again for providing us the opportunity to improve our manuscript. We hope that these revisions are adequate, and that our manuscript could be eligible for further processing and evaluation by the reviewers.

Sincerely,

Prof Min Gu

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Response to Reviewer 2 (in detailed)

Dear reviewers:
Thanks a lot for you to review our manuscript and raised many valuable comments towards our paper. We have made some corrections and promoted our manuscript according to these comments, which are shown as follows:

Comments for the Authors: The manuscript entitled "A genetic polymorphism of CYP3A4 rs2242480 associated with sirolimus trough concentrations among adult renal transplant recipients" has merit, may be accepted after fulfilling the following major concerns:

1. **Authors must consider proof checking for English by any native English speaker; there is a lack of flow at various places; simultaneously disconnect between lines/sentences were also evident.**
   Response: We have completed a native professional proof reading for English. We also attached the proof reading certificate.

2. **Don't use abbreviations before providing the full name and defining them first. Then, always use the abbreviation.**
   Response: We received your suggestion and will use the abbreviation.

3. **Adding an abbreviation list will be an added value.**
   Response: We received your suggestion, and attached the list of abbreviations after the section of "Conclusion".
   Revision :

   List of abbreviation
   ABCB : ATP- binding cassette sub family B
   ANOVA : analysis of variance
   AR : acute rejection
   BID : bis in die
   BMI : body mass index
   Chr : chromosome
   CsA : cyclosporine
   CYP450 : cytochrome P-450
   DGF : delayed graft function
   DNA : deoxyribonucleic acid
   EDTA : ethylene diamine tetraacetic acid
   gDNA : genomic DNA
   GLMs : general linear models
   HIV : human immunodeficiency virus
   HSD : hydroxysteroid dehydrogenases
   HWE : hardy-weinberg equilibrium
   IL-10 : Interleukin 10
   LD : linkage disequilibrium
   MAF : minor allele frequency
   MMF : mycophenolate mofetil
   NR : nuclear receptor
   PK : pharmacokinetic
   POR : P-450 oxidoreductase
   PPARA : peroxisome proliferator activated receptor alpha

Pred : prednisone
PXR : pregnane x receptor
RLU : relative light unit
Scr : serum creatinine
SD : standard deviation
SIR : sirolimus
SNPs : single nucleotide polymorphisms
TAC : tacrolimus
TS : target sequencing
UG/T : uridine 5′-diphospo-glucuronosyl transferase
UTR : untranslated region

4. **In the last paragraph of the "Statistical analysis" section, the P-value was set to 0.1, why? And why not 0.05 as usual?**
Response: Considering to the limited case number included in this study and potential various confounding factors involved, we attempted to obtain more significant SNPs derived from logistic regression analysis for further association analysis. However, even if we had expanded the *P* value into 0.1, only one SNP was found to be significantly associated with the sirolimus metabolism after 12 months.

5. **Also, In general, the level of significance should be corrected for multiple testing. The authors should clarify this point.**
Response: In our study, we performed logistic regression analysis adjusted by significant confounding factors, limited in one statistical model, to explore the significant SNPs associated with the sirolimus concentrations in renal transplant recipients, which should be considered as one testing instead of multiple testing. Thus, we felt so appreciated for your considerations here and there was no need of the correction for multiple testing among the statistical analysis.

6. **Many references are missing such as the one for Haploview 4.2 and also Tagger.**
Response: We have attached the corresponding references for crucial techniques and statistical analysis, including Haploview 4.2 and also Tagger. Please refer to the section of "Methods and Materials".
Revision :

- All analyses were based on the UCSC build hg19 human reference sequence (NCBI build 37.2) using Burrows-Wheeler [28]. Besides, suspected somatic variants suspected detected by MuTect 1.1.5 and VarScan 2.3.6, were identified by pairing each sample with the corresponding blood sample [29,30].
- Minor allele frequencies (MAF), Hardy-Weinberg equilibrium (HWE), and linkage disequilibrium LD) were determined using Haploview 4.2 (Broad Institute, MA, Cambridge, USA) [31]
- We identified 80 SNPs with HWE and MAF ≥0.05, and selected 36 tag SNPs for single-site analysis. Tag SNPs that capture information on other variants with MAF ≥0.05 were selected using the Tagger program (BROAD Institute, implemented in Haploview) [34].

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[30] Koboldt, D.C.; Zhang, Q.; Larson, D.E.; Shen, D.; McLellan, M.D.; Lin, L.; Miller, C.A.; Mardis,

E.R.; Ding, L.; Wilson, R.K. VarScan 2: Somatic Mutation and Copy Number Alteration Discovery in Cancer by Exome Sequencing. *Genome Res.*, **2012**, *22*, 568–576.
[31] Barrett, J.C.; Fry, B.; Maller, J.; Daly, M.J. Haploview: Analysis and Visualization of LD and Haplotype Maps. *Bioinformatics*, **2005**, *21*, 263–265.
[34] de Bakker, P.I.W.; Yelensky, R.; Pe'er, I.; Gabriel, S.B.; Daly, M.J.; Altshuler, D. Efficiency and Power in Genetic Association Studies. *Nat. Genet.*, **2005**, *37*, 1217–1223.

7. **"From the Table 1, we found that most recipients were males (73.91%) with a mean ± SD of weight and age at 63.22 ± 10 kg; 35.22 ± 10 years." From this sentence, the reader will understand that these numbers belong to males only, while returning to Table 1, these numbers are for the whole cases (males and females). Please rewrite adequately.**

Response: We have rewrite it adequately.

Revision :

Table 1 shows that most recipients were males (73.91%) while females only (26.09%). The mean (± SD) weight and age of participants was 63.22 ± 10 kg; 35.22 ± 10 years, respectively.

8. **In Table 1, for Gender and Type of donor parameters, only (n) was represented while (%) values are missing.**

Response: We have revised it.

Revision :

Table 1. Baseline clinical characteristics in the cohort.

Clinical Variables	Value
Case number (n)	69
Age (years; mean ± SD)	35.22 ± 10
Gender, n (%)	
Male	51 (73.91)
Female	18 (26.09)
Weight (kg, mean ± SD)	63.22 ± 10
Duration after renal transplant (days, mean ± SD)	1964 ± 674
PRA before renal transplant (%)	0
Primary/secondary renal transplant	69/0
Type of donor, n (%)	
DCD	64 (92.76)
Living-related	5 (7.25)
ISD protocol	
Prednisone + MMF + Tacrolimus	37
Prednisone + MMF + CsA	32
Incidence of DGF episodes, n (%)	25 (36.23)
Incidence of AR episodes, n (%)	37 (53.62)

9. **In Table 1, please arrange the abbreviations alphabetically.**

Response: The abbreviation has arranged alphabetically.

Revision :

Abbreviations: AR, Acute Rejection; CsA, Cyclosporine; DCD, Donation after Cardiac Death; DGF, Delayed Graft Function; ISD, immunosuppressive drugs; MMF, Mycophenolate Mofetil; PRA, Panel Reactive Antibodies; SD, Standard Deviations.

10. Supplemental Table 1 was not mentioned in the whole article.

Response: We have revised for Suplemental Table 1

Revision :

Targeted sequencing (TS) analysis based on next-generation sequence obtained a total of 300 SNPs for all genes (*CYP3A4*, *CYP3A5*, *CYP2C8*, *CYP2C19*, *POR*, *PPARA*, *ABCB1*, *HSD11B1*, *NR3C1*, *UG/T1A8*, *UG/T2B7* and *UG/T1A9*) including 20 SNPs in *CYP3A4*, 20 SNPs in *CYP3A5*, 27 SNPs in *CYP2C8*, 27 SNPs in *CYP2C19*, 63 SNPs in *POR*, 18 SNPs in *PPARA*, 58 SNPs in *ABCB1*, 8 SNPS in *HSD11B1*, 22 SNPs in *NR3C1*, 8 SNPs in *UG/T1A8*, 4 SNPs in *UG/T1A9*, 22 SNPs in *UG/T2B7* and 4 novel SNPs with undetermined genotype names (Supplemental Table 1).

11. There are three methods in Haploview that create haplotype blocks. Which method was used to detect the 15 haplotype blocks mentioned in this article?

Response: In our study, we defined the block by confidential intervals (Gabriel et al). In detail, confidential interval minimal for strong LD was (0.7, 0.98); upper confidential interval maximum for strong recombination was 0.9; Fraction of strong LD in informative comparison must be at least 0.95; and exclude makers below 0.05 MAF.

12. The quality of Supplemental Figure 1 is too poor. The block numbers, block sizes, rsID cannot be read.

Response: We have modified the Supplemental Figure 1, and uploaded the revised one.

13. The link between the haplotype blocks in Supplemental Figure 1 and gene names in the article cannot be traced.

Response: In Supplemental Figure 1, headings above each SNP loci represent their location on difference chromosome. In this study, we used target sequencing technology based on second-generation sequencing, and some novel SNPs were reported without any previous report and nomination. Therefore, we have to use each location to show each SNP loci here.

14. This reviewer thinks that Supplemental Tables are shifted till Supplemental Table 4.

Response: we have revised the order of Suplemental Tables

Revision :

Targeted sequencing (TS) analysis based on next-generation sequence obtained a total of 300 SNPs for all genes (*CYP3A4*, *CYP3A5*, *CYP2C8*, *CYP2C19*, *POR*, *PPARA*, *ABCB1*, *HSD11B1*, *NR3C1*, *UG/T1A8*, *UG/T2B7* and *UG/T1A9*) including 20 SNPs in *CYP3A4*, 20 SNPs in *CYP3A5*, 27 SNPs in *CYP2C8*, 27 SNPs in *CYP2C19*, 63 SNPs in *POR*, 18 SNPs in *PPARA*, 58 SNPs in *ABCB1*, 8 SNPS in *HSD11B1*, 22 SNPs in *NR3C1*, 8 SNPs in *UG/T1A8*, 4 SNPs in *UG/T1A9*, 22 SNPs in *UG/T2B7* and 4 novel SNPs with undetermined genotype names (Supplemental Table 1).

When considering all 69 recipients, 80 SNPs with MAF≥0.05 were included in further single site association analysis (Supplemental Table 2).

We found 36 tag SNPs, including 8 SNPs in *ABCB1*, 4 SNPs in *CYP2C19*, 4 SNPs in *CYP2C8*, 1 SNP in *CYP3A4*, 3 SNPs in *CYP3A5*, 1 SNP in *HSD11B1*, 3 SNPs in *NR3C1*, 5 SNPs in *POR*, 2 SNPs in *PPARA*, 3 SNPs in *UG/T1A8*, and 2 SNPs in *UG/T2B7* were examined (Supplemental Table 3).

We analysed the clinical variables influencing the SIR trough concentrations in the combined affect analysis using a multivariable GLM. Clinical factor such age (P=0.999), follow up duration (P=1), incidence of DGF (P=0.999), immunosuppressant protocol (P=0.991), sex (P=0.987) and AR (P=0.954) showed no significant association with SIR C0/Dose/BMI levels using those models (P>0.1) (Supplemental Table 4).

15. At the end of the second paragraph of the "Discussion" section, the P value threshold was 0.01,

while previously in the text, the P value threshold was 0.1. Something is wrong.

Response: We feel so sorry for our mistake here. It should be corrected from 0.01 into 0.1.

Revision:

All of these genes have an important role in determining the therapeutic outcome of drug levels in the blood. The GLM showed that 4 SNPs had a significant effect on the SIR steady state concentration measured after 1 year of transplantation : *CYP3A5* (Chr7: 99260362 C>A, rs4646453 and Ch7: 99245914 A> G, rs15524), *CYP3A4* (Ch7: 99361466 C>T, rs2242480) and *PPARA* (Chr22: 46615625 G> A, rs1800246) (P<0.1).

We analysed the relationship between each SNP and 12 months of SIR PKs to observe a significant difference. GLM analysis showed that 4 SNPs positioned in Chr7:99361466 intronic C>T rs2242480 (P=0.008, *CYP3A4*), Chr22:46615625 intronic G>A rs1800246 (P=0.077, *PPARA*), Chr7:99245914 UTR3 A>G rs15524 (P=0.026, *CYP3A5*) and Chr7:99260362 intronic C>A rs4646453 (P=0.036, *CYP3A5*) were significantly associated with the dose- and BMI- normalized SIR concentrations (P<0.1).

We sincerely hope that these revisions are adequate, and that our revised manuscript is now acceptable for publication in "*Current Drug Metabolism*" Journal.

Best regards!

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