Author's response to reviews

Title: C-reactive protein genetic variant is associated with diabetic retinopathy in Chinese type 2 diabetic patients

Authors:

Danfeng Peng (pdandanf@sjtu.edu.cn)
Jie Wang (jiediana113@126.com)
Rong Zhang (rongzhang11@hotmail.com)
Shanshan Tang (tss8235078@163.com)
Feng Jiang (jiangfeng1031@hotmail.com)
Miao Chen (chenmiao2011@126.com)
Jing Yan (yanjing@sjtu.edu.cn)
Xue Sun (443782999@qq.com)
Tao Wang (502472450@qq.com)
Shiyun Wang (695107677@qq.com)
Yuqian Bao (byq522@163.com)
Cheng Hu (alfredhc@sjtu.edu.cn)
Weiping Jia (wpjia@sjtu.edu.cn)

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Author's response to reviews: see over
Dear Editor and reviewer,

Firstly we want to thank you for your kind positive comments about our work and for the critical reading and critiques of this manuscript, which we found to be extremely helpful in our revising and improving this manuscript. These critiques are addressed as follows and as highlighted in the revised manuscript.

Thank you again for all the efforts in helping improve this manuscript.

Sincerely,
Weiping Jia, M.D., Ph.D.
Director, Professor of Medicine
Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth People’s Hospital, Shanghai, China
Editor's Comments:

"The reviewers are of mixed opinion on this manuscript. The role of inflammation and vascular complications is not a new one (King et al, Brownlee et al, Aiello et al, Krolewski et al, Orchard et al), but has not been well-studied in an East Asian population and is increasingly important due to the epidemic of diabetes in this population. Dr. Narne's comment regarding the presentation of a hypothesis regarding the role of the allelic change would add significantly to the paper. Please add to the discussion what amino acid/ conformational change this SNP lead to."

Response: Thank you for your comments. In our study, we found rs2808629 was significantly associated with DR in Chinese patients with T2DM. As we described in the "discussion", this SNP locates in the downstream of CRP and it does not lead to amino acid change. It was reported to be associated with serum CRP level in a GWAS. We hypothesize rs2808629 may participate in the susceptibility of DR through its effects on regulating CRP expression or it may be just a genetic marker in linkage disequilibrium with the causal variant(s). However, further studies are warranted to reveal the underlying mechanism.

Referee 1
Reviewer: Parimala Narne
Reviewer's report:
Major compulsory revisions
The paper needs editing as there are grammatical errors and inaccurate tense.
Introduction:
• The allelic composition of the proposed SNP loci needs to be elaborated.

Response: In our study, tagging SNPs with CRP region were selected. We described the details in the “Single nucleotide polymorphisms (SNPs) selection, genotyping and quality control” section.

• The documented pathological relevance of CRP in DR has to be specified.

Response: Thank you for your suggestion. We have modified the second paragraph of “introduction” section. We wrote, “C-reactive protein (CRP), a very sensitive marker of inflammation produced by the liver cells in response to various stimuli, is involved in endothelial dysfunction and atherogenesis which have been proposed to play an important role in the pathogenesis of DR”.

• The possible complicity of the respective genetic variants in development/progression of DR needs to be specified.

Response: According to your suggestion, we have added “To data, although no locus for DR from genome-wide association study (GWAS) reaches conventional significance
criteria, but a significant number of genes and genetic variants have been proposed for DR or proliferative DR through candidate gene approach [10, 11]. Several pathways and processes, including the renin-angiotensin system, vascular endothelial dysfunction, tissue matrix remodeling, and angiogenesis, have been strongly implicated in the pathogenesis of DR, and multiple genes involved in these pathways have been identified for DR (e.g., AKR1B1, VEGFA, ACE, and AGER)” in the first paragraph of “introduction” section.

• It is necessary to elucidate the biological plausibility of the investigated genetic variants.

Response: we selected tagging four SNPs within CRP region. Rs2808634 locates in the upstream of CRP; rs1130864 locates in the 3’ UTR of CRP; rs2808629 and rs3093077 locate the downstream of CRP. Since none of these four SNPs locates in the coding region of CRP, the investigated SNPs are just genetic markers, so we did not describe it in the “introduction” section.

Materials and methods:
• The characteristic symptoms of different groups of DR in terms of varying degree of severity viz., NPDR, PDR etc., have not been precisely enumerated. Further, the term ‘worse eye’ is ambiguous.

Response: we appreciate your advices. In our study, retinopathy was graded according to the International Classification of Diabetic Retinopathy according to this reference “Wilkinson CP, et al. Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. Ophthalmology 2003, 110:1677-1682”. The characteristic symptoms of different groups of DR are shown in the table as follow:

<table>
<thead>
<tr>
<th>Proposed Disease Severity Level</th>
<th>Findings Observable on Dilated Ophthalmoscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>No apparent retinopathy</td>
<td>No abnormalities</td>
</tr>
<tr>
<td>Mild nonproliferative diabetic retinopathy</td>
<td>Microaneurysms only</td>
</tr>
<tr>
<td>Moderate nonproliferative diabetic retinopathy</td>
<td>More than just microaneurysms but less than severe nonproliferative diabetic retinopathy</td>
</tr>
<tr>
<td>Severe nonproliferative diabetic retinopathy</td>
<td>Any of the following: more than 20 intraretinal hemorrhages in each of 4 quadrants; definite venous beading in 2+ quadrants; Prominent intraretinal microvascular abnormalities in 1+ quadrant And no signs of proliferative retinopathy</td>
</tr>
<tr>
<td>Proliferative diabetic retinopathy</td>
<td>One or more of the following: neovascularization, vitreous/preretinal hemorrhage</td>
</tr>
</tbody>
</table>
And we have modified the “worse eye”. We wrote, “For each eye, a DR grade was assigned. A subject’s DR grade was based on the level of the more severe eye”.

• There is no mention about usage of a standard questionnaire for collection of detailed information regarding anthropometric measurements.

Response: Thank you for your suggestion. We have added “Each participant completed a standard questionnaire for detailed information as described previously [20]” in “Clinical measurement” section.

• The standard definition of BMI and the cut off values of SBP and DBP for deeming the patients hypertensive have not been provided.

Response: We have added the standard definition of BMI and the definition of hypertension in the “method” section. We wrote, “Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared” and “Hypertension was defined as systolic blood pressure \( \geq 140 \) mmHg and/or diastolic blood pressure \( \geq 90 \) mmHg”.

• The distinctive number of the T2DM subjects allocated to the respective categories of DR severity has not been mentioned.

Response: Thank you for your suggestion. According to your recommendation, we have added a table titled “CRP SNPs distribution among patients with different severity of DR” in the manuscript.

• It needs to be made clear as to whether binary/multiple logistic regression analysis has been employed for statistical analysis of genotypic and epidemiological data.

Response: Multiple logistic regression analysis was used in our statistical analysis. We have added it in “Statistical analysis” section.

Tables
• The proportion of the T2DM subjects with/without hypertension has not been indicated.

Response: Thank you for your suggestion. We have added “subjects with hypertension” in Table 1.

• Despite it being mentioned that the DR cases have been categorized in terms of varying degree of severity, there is no mention of the frequency distribution of alleles and genotypes in the respective categories.

Response: We have added the information about DR severity in the second paragraph of the “Results” section. We wrote, “Of the 593 DR patients, there were 379 patients with mild NPDR, 98 with moderate NPDR, 80 with severe NPDR and 36 with PDR. The
distribution of these four SNPs among different levels of DR was shown in Table 3”.
And a table titled “CRP SNPs distribution in patients with different severity of DR” has been added in the revised manuscript.

Discussion

• It would be more convincing to concentrate on the relationship between CRP and DR, in lieu of the microvascular complications in general.

Response: Thank you for your suggestion. We have modified the “discussion” section and emphasized demonstrating the relationship between CRP and DR.

• It would be more reasonable to provide a cogent explanation for the observed association between CRP variant and DR.

Response: As we described in “discussion”, rs2808629, the SNP associated with DR in our study, locates in the downstream of CRP. It was reported to be associated with serum CRP level in a GWAS. We hypothesize rs2808629 may participate in the susceptibility of DR through its effects on regulating CRP expression or it may be just a genetic marker in linkage disequilibrium with the causal variant(s). However, further studies are warranted to reveal the underlying mechanism. This is also one of limitations of our study.

• It is very much required to comment on the power of the study.

Response: We have added power calculation results at the end of “statistical analysis” section. We wrote, “On the basis of the previously reported effect size of genetic loci for DR (~1.40), our samples had > 90% power to detect an effect SNP with minor allele frequency of 0.3 at a 0.05 level and > 80% power to detect an effect SNP with minor allele frequency of 0.2 at a level of significance of 0.05”.

• It would be ideal to discuss the significance of the study and provide future perspectives as part of conclusions as it appears abrupt in its current form.

Response: Thank you for your suggestion. We have improved the “conclusions” section. We wrote, “This study discover the association of CRP variant with DR for the first time, although the mechanism underlying the genotype–phenotype association is unknown, it provide a hypothesis for future researches. Further studies are needed to replicate this finding in other populations and translate the common variant association signal into biological mechanisms of disease causation”.

Minor Essential Revisions
• Line 137: ‘Microalbuminuria’ has been misspelt.

Response: A according to your suggestions, we focused on discussing the relationship between CRP and DR. So this sentence has been deleted. We have also checked the whole
Further, what were the risk factors that emerged as significant ones in determining the risk for DR in the logistic regression analysis?

Response: In our logistic regression analysis model, duration of diabetes, HbA1c and systolic blood pressure were risk factors for DR.

Referee 2
Reviewer: Daniel Petrovic
Reviewer's report:

MAJOR COMPULSORY REVISION

The MS “C-reactive protein genetic variant is associated with diabetic retinopathy in Chinese type 2 diabetic patients” is rather well written research paper. In their study on a large sample of cases and controls they demonstrated that CRP rs2808629 was associated with DR in the Chinese type diabetic patients.

Abstract is informative, well written.

Introduction section should be improved.
Comment 1: At the end of the first paragraph of the Introduction section (page 3, after lines 51, 52) the authors should mention the importance of genetic factors before starting with CRP. Nice nice review of candidate genes for DR is below (ref 1)

Response: Thank you for your suggestions. At the end of the first paragraph of the Introduction section, we wrote, “To date, although no locus for DR from genome-wide association study (GWAS) reaches conventional significance criteria, but a significant number of genes and genetic variants have been proposed for DR or proliferative DR through candidate gene approach [10, 11]” and cited this nice review.

Comment 2: In the aim of the study (page 4, line 63) the authors should omit the phrase “after adjustment for established conventional risk factors”. It is not necessary.

Response: Thanks for your suggestion. We have deleted the phrase “after adjustment for established conventional risk factors”.

Methods section is generally OK, although few details should be improved.
Comment 3: In the first paragraph of the Methods section (page 4, line 3) the newer criteria of the T2DM should be used (see below) instead of WHO from 1999 American Diabetes Association.
Response: Thank you for your suggestion. Subjects of this study were selected from the inpatient database of Shanghai Diabetes Institute which enrolled participants from the year of 2006 to 2008. The data of HbA1c were incomplete, so 1999 WHO criteria were used for diagnosing T2DM. Besides, the cutoff value of HbA1c for diagnosing diabetes in Chinese population is uncertain now.

Comment 4: In the first paragraph of the Methods section, Participants (page 4, line 3) the authors wrote that the patients were unrelated. How was this evaluated? Genetically or just according to personal history?

Response: In our study, it was according to personal history to ensure the subjects were unrelated.

Comment 5: In the Methods section the authors should write whether they used Bonferroni correction for multiple testing. If not, than it would be appropriate to use it.

Response: We did not use Bonferroni correction for multiple testing. Instead, correction for multiple testing was performed using Haploview through 10,000 permutation tests. Empirical $P$ values in Table 2 were calculated based on 10,000 permutations.

Discussion section should be improved.
Comment 6: In the first paragraph of the Discussion section the authors should start with the answer to the aims of the study, and not to start will secondary introduction.

Response: Thank you for your suggestion. We have rearranged the structure of the “discussion” section.

Comment 7: In the whole manuscript the authors should omit the expression “diabetic”: in diabetic population, diabetic patients They should be for example: in subjects with diabetes mellitus or subjects with T2DM or something similar

Response: Thank you for your advice. We have checked the whole manuscript and changed “diabetic” to proper expression.

Comment 8: In the last paragraph of the Discussion section (Conclusions) the authors should improve the sentence: Further studies are needed to replicate this finding in other populations.

Response: Thank you for your advice. We have improved this sentence.

Comment 9: Language should be improved (native speaker or expert for English language).
Referee 3
Reviewer: Monika Buraczynska
Reviewer's report:
Major Compulsory Revisions:
1. In the Methods section: The information given about study groups is too sparse. The exact inclusion / exclusion criteria for the patient population should be given. In the study population 618 patients (61 %) had diabetic retinopathy. This number is much higher than the prevalence of DR reported for Chinese population. Does that mean that T2DM patients were selected for diabetic retinopathy and not randomly selected? If so, it should be stated in the paper. There is no mention how many patients had diabetic nephropathy or whether there were any patients without DR but with DN? This is very important and could affect the interpretation of results. Also, there is no information on macrovascular complications in the study subjects.

Response: According to your advice, in the “Participants” section, we wrote, “All participants were unrelated patients with T2DM meeting the 1999 WHO criteria (fasting plasma glucose ≥ 7.0 mmol/l and/or 2 h plasma glucose ≥ 11.1 mmol/l). Type 1 diabetes and mitochondrial diabetes were excluded by clinical, immunological (individuals with GAD and/or protein tyrosine phosphatase IA-2 antibodies were excluded) and genetic methods (mitochondrial tRNA^Leu(UUR) A3243G mutation carriers were excluded). “

For the selection for participants, patients with T2DM for over 10 years but without DR were selected as controls for DR. So 61% did not represent the prevalence of DR.

According to your suggestion, we have added “subjects with nephropathy” in Table 1. And we analyzed the association of SNPs with DR adjusting duration of diabetes, HbA1c, systolic blood pressure, diastolic blood pressure, body mass index, sex and nephropathy, the results were similar as those without adjusting nephropathy (showed as below).

<table>
<thead>
<tr>
<th>SNP</th>
<th>OR (95%CI)</th>
<th>P value</th>
<th>OR (95%CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2808629</td>
<td>1.261 (1.022, 1.555)</td>
<td>0.030</td>
<td>1.278 (1.035, 1.579)</td>
<td>0.023</td>
</tr>
<tr>
<td>rs3093077</td>
<td>1.194 (0.906, 1.574)</td>
<td>0.209</td>
<td>1.212 (0.918, 1.601)</td>
<td>0.176</td>
</tr>
<tr>
<td>rs1130864</td>
<td>1.250 (0.794, 1.968)</td>
<td>0.335</td>
<td>1.263 (0.800, 1.995)</td>
<td>0.317</td>
</tr>
<tr>
<td>rs2808634</td>
<td>1.056 (0.804, 1.386)</td>
<td>0.697</td>
<td>1.063 (0.809, 1.397)</td>
<td>0.661</td>
</tr>
</tbody>
</table>

* adjusted for duration of diabetes, HbA1c, systolic blood pressure, diastolic blood pressure, body mass index, and sex
§ adjusted for duration of diabetes, HbA1c, systolic blood pressure, diastolic blood pressure, body mass index, sex and nephropathy

However, we lack the data of macrovascular complications in our study subjects. It is a limitation of our study.
2. The Results section of the manuscript is not very informative. For example, apart from the note that clinical characteristics of the samples (shouldn’t it be rather “patients” or “subjects”?) are shown in Table 1, there is no comment anywhere on this comparison between DR+ and DR- patients. Even in the table itself, they don’t indicate whether the differences were statistically significant (p values?).

Response: Thank you for your suggestions. We have changed “samples” to “patients” in Table 1. We also compared the characteristics between DR+ and DR- patients and added details in the first paragraph of the Results section. We wrote, “Compared with patients without DR, patients with DR were diagnosed with diabetes at earlier age and had higher HbA1c levels and higher prevalence of hypertension and diabetic nephropathy. Besides, because patients without DR selected in our study had diabetes for over 10 years, they were older and had longer duration of diabetes compared with patients with DR”. In Table 1, P values have been added too.

3. Not all data are presented in the tables or even described in the text. The Authors claim that they examined the effect of rs2808629 on the DR severity. These results should be presented in the manuscript. How many patients had proliferative DR?

Response: We have added the information about DR severity in the second paragraph of the “Results” section. We wrote, “Of the 593 DR patients, there were 379 patients with mild NPDR, 98 with moderate NPDR, 80 with severe NPDR and 36 with PDR. The distribution of these four SNPs among different levels of DR was shown in Table 3”. And Table 3 named “CRP SNPs distribution among patients with different severity of DR” has been added. The detail information about rs2808629 and DR severity was showed as below:

<table>
<thead>
<tr>
<th>group</th>
<th>rs2808629</th>
<th>MAF</th>
<th>P for trend analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients without DR</td>
<td>141 180 57</td>
<td>0.389</td>
<td></td>
</tr>
<tr>
<td>Mild NPDR</td>
<td>110 180 84</td>
<td>0.465</td>
<td></td>
</tr>
<tr>
<td>Moderate NPDR</td>
<td>25 52 18</td>
<td>0.463</td>
<td></td>
</tr>
<tr>
<td>Severe NPDR</td>
<td>25 44 10</td>
<td>0.405</td>
<td></td>
</tr>
<tr>
<td>PDR</td>
<td>11 21 3</td>
<td>0.386</td>
<td></td>
</tr>
</tbody>
</table>

4. Table 1 has some shortcomings. Sample sizes, 593 for DR+ patients and 388 for DR-, are different from given in the Abstract and Participants subsection of Methods (618 for DR+ and 400 for DR-). Although it is stated somewhere that 37 samples were excluded, this information should also be given in the footnotes for the Table 1. For the comparison of DR+ with DR-, the p-values should be included. For BMI the units should be kg/m2 not years!
Response: Thank you for your suggestions. “25 patients with DR and 12 patients without DR were excluded due to sample call rate check” has been added in the footnotes for Table 1. $P$ values have been added in Table 1 too. The units of BMI have been corrected.

Minor Essential Revisions:
1. The results of the Hardy-Weinberg equilibrium test should be shown.

Response: We have added the results of the Hardy-Weinberg equilibrium test in the “Single nucleotide polymorphisms (SNPs) selection, genotyping and quality control” section. We wrote, “The Hardy-Weinberg equilibrium test was performed before the association analysis, and all the four SNPs were in accordance with Hardy-Weinberg equilibrium ($P = 0.68$ for rs2808629, $P = 0.74$ for rs3093077, $P = 0.34$ for rs1130864 and $P = 0.47$ for rs2808634, respectively)”.

2. The Discussion section is more a summary of a literature, rather than the proper discussion of the study results. The Authors should include some more comments on the biological role of CRP relevant to diabetes and its complications. Also, some useful papers could be added for the discussion purpose, for example Lim et al. IOVS 2010; 51 (9): 4458.

Response: Thank you for your suggestions. The first reviewer also gives similar advice on the “discussion”. This paper is very useful for us. And we have modified the “discussion” section according to your suggestions.

3. Mentioning the limitations of the study, the Authors say that their sample size was relatively small, so the association detected could be a false positive. That’s why the power calculation results should be described in the Methods or Results. What would be the sample size required to observe a true effect?

Response: According to your suggestion, we have added power calculation results at the end of “statistical analysis” section. We wrote, “On the basis of the previously reported effect size of genetic loci for DR (~1.40), our samples had > 90% power to detect an effect SNP with minor allele frequency of 0.3 at a 0.05 level and > 80% power to detect an effect SNP with minor allele frequency of 0.2 at a level of significance of 0.05”. And we also have modified related part in the “discussion” section. Considering the power of our study, the possibility of a false positive is limited. However, further studies are needed to replicate our results.

Discretionary Revisions:
1. In this study the Authors did not include a control group of non-diabetic subjects to assure that the frequencies of the genotypes and alleles of the rs2808629 polymorphism in CRP gene are similar to reported in other studies of Asian populations. This would indicate that no technical errors were present.
Response: We have genotyped rs2808629 in 438 non-diabetic subjects. In the HapMap database for rs2808629, there are 3 studies of Asian populations. Distribution of rs2808629 in our study is similar to those reported in other studies of Asian population. The frequencies of the genotypes and alleles of rs2808269 in these studies are shown as the table below.

<table>
<thead>
<tr>
<th>Study</th>
<th>Samples</th>
<th>AA frequencies</th>
<th>AG frequencies</th>
<th>GG frequencies</th>
<th>A allele frequencies</th>
<th>G allele frequencies</th>
</tr>
</thead>
<tbody>
<tr>
<td>HapMap-HCB</td>
<td>86</td>
<td>0.326</td>
<td>0.512</td>
<td>0.163</td>
<td>0.581</td>
<td>0.419</td>
</tr>
<tr>
<td>HapMap-JPT</td>
<td>172</td>
<td>0.512</td>
<td>0.384</td>
<td>0.105</td>
<td>0.703</td>
<td>0.297</td>
</tr>
<tr>
<td>HapMap-HCB</td>
<td>82</td>
<td>0.220</td>
<td>0.488</td>
<td>0.293</td>
<td>0.463</td>
<td>0.537</td>
</tr>
<tr>
<td>Our study</td>
<td>438</td>
<td>0.295</td>
<td>0.505</td>
<td>0.201</td>
<td>0.547</td>
<td>0.453</td>
</tr>
</tbody>
</table>

2. The English language should be carefully edited.

Response: We modified the manuscript, hope it is better now.