Author's response to reviews

Title: A Novel Multiplex Polymerase Chain Reaction Assay for Profile Analyses of Gene Expression in Peripheral Blood

Authors:

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Author's response to reviews: see over
Dear Editors and Reviewers:

Thank you for your letter and for the reviewers’ comments concerning our manuscript entitled “A Novel Multiplex Polymerase Chain Reaction Assay for Profiles Analysis of Peripheral Blood Gene Expression”. (ID: 1876195980645702). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied comments carefully and have made correction. The revised portions are marked in blue in the paper. The main corrections in the paper and the responds to the reviewer’s comments are as flowing.

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and make the manuscript smooth. And we answer the reviewer’ question point by point.

We appreciate for Editors and Reviewers’ warm work earnestly, and hope that the correction will meet with approval.

Looking forward to hearing from you.

Best wishes

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Responds to the reviewer’s comments:

Reviewer #1: James Wingrove

**Question**
The authors validated each primer pair individually for appropriate size (Figure 1 and Table 1); it would be worthwhile to also report the efficiencies of the assays using a standard curve, both singularly as well as when multiplexed.

Response: Thanks for the reviewer’s suggestion and it is really true that to evaluate the efficiencies of one assay using a standard curve and it is necessary for a quantitative test. However, our assay is a semi-quantitative test and each test contains housekeeping gene. Through the ratios of the target gene and housekeeping gene we can get the relative expression. If the efficiencies of the assay are different between different tests, it will affect both testing genes and housekeeping gene simultaneously, and the ratios will not be influenced.

**Question**
On page 8 the authors state the reverse primer concentrations for 8 genes need to be either increased or decreased and show the results of these alterations in Figure 2. Figure 2 is of poor quality and uninterpretable (as is Figure 1); it would also be beneficial to summarize the results of the optimization in tabular form, quantifying the decrease or increase in peak height as a ratio to the positive control.

Response: Thanks for the reviewer’s valuable comments and the figure 2 is replaced by a high of quality and the peak height as a ratio to the positive control have been summarized in tabular form to show the decrease or increase in peak height as a ratio to the positive control.
<table>
<thead>
<tr>
<th>gene/SELL(2)</th>
<th>gene/SELL(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1B</td>
<td>1.86</td>
</tr>
<tr>
<td>ACTB</td>
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<tr>
<td>VWF</td>
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</tr>
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<td>IL6</td>
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<tr>
<td>MTHFR</td>
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<td>SELL(1)</td>
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<tr>
<td>MCP-1</td>
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<tr>
<td>INFN</td>
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<tr>
<td>TNFalpha</td>
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<tr>
<td>GK</td>
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<td>IL8</td>
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<td>MCSF</td>
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<td>ICAM1</td>
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<tr>
<td>ID2</td>
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</tr>
<tr>
<td>LDLR</td>
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<tr>
<td>HMOX1</td>
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<tr>
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</tr>
<tr>
<td>KanR</td>
<td>0.24</td>
</tr>
</tbody>
</table>

**Question**

As the samples are collected into tubes without RNA stabilization reagents, concern exists around the length of time prior to RNA purification; have the authors evaluated the effects of time on RNA stability?

**Response:** Thanks for the reviewer’s suggestion and we have compared different time of the samples in tubes without RNA stabilization reagents. The result showed that RNA could be stable within 6 hours in sample tubes at room temperature. Actually, during our experiments the RNA was extracted as soon as possible and usually within 2 hours.

**Question**

The number of patients evaluated was rather low, it would be beneficial to see the results replicated in an independent set of patients to confirm the findings.
Response: Thanks for the reviewer’ valuable comments and another 30 subjects have recruited to validate the previous results. The results indicated that the p values of IL-1β, IL-6, IL-8 and MCP-1 between these two groups were 0.023, 0.014, 0.021, and 0.048 respectively.

Question

As the patients in the study were evaluated by CT-angiography, it should be possible to define the amount of disease present in the cases; the disease severity here is unclear. Assuming there is a mixed amount of disease in this set of patients (ranging from patients with low burden to multi-vessel disease) it would be interesting for the authors should look at the levels of gene expression versus amount of disease (including controls).

Response: Thanks for the reviewer’ valuable suggestions. There are enough evidence suggested that many acute coronary syndromes are caused by plaque disruption and thrombosis rather than stenosis severity. The composition and configuration of atherosclerotic plaques are the main factors for plaque stability. According to the CT findings, subjects of Group A were designed as those without coronary atherosclerotic plaque in any branch of coronary arteries. And Group B included the patients with totally calcified plaques in any coronary artery, no macroscopic noncalcified plaque. And Group C included the subjects with noncalcified plaque and mixed plaque (a plaque contained both calcified and noncalcified components). (Plaque classified criteria was described as the manuscript.) And the CT findings of Group B and C were regarded as coronary atherosclerosis. It is really true as Reviewer suggested that there is a mixed amount of disease in this set of patients, the aim of this study was to compare the gene expression variation between different study groups which could provide some information related with the properties of the artery plaque.

Question

Gene expression is known to be strongly associated with age, sex, and other clinical
risk factors. The authors need to evaluate these in relationship to the changes that are being observed (e.g. multivariate analysis); the case and control sets were not completely balanced for age and sex.

Response: Thanks for the reviewer’s valuable comments. We are sorry that the list of vascular risk factors has not been provided in the first manuscript. The clinical characteristics, such as hypertension, diabetes mellitus, hyperlipidemia, and smoking have been showed in Table 1 in the revised manuscript. There were no statistic difference (P > 0.05), including age and sex were balanced.

Question
In the section describing precision results for gene expression, it is unclear what the range of CV’s given represent. Is this the range seen within the individual genes? Also, reporting the results as CV is not entirely correct, as gene expression is a quantitative measurement using an interval scale; using SD is more appropriate.

Response: Thanks for the reviewer’s comments. There are a total of 18 peaks (Kanamycin RNA is exclude) and the precision is represented as the average percentage CV for all of the peaks within this panel. The CV’s ranges refer to lowest and highest result. It is really true as reviewer suggested that reporting the gene expression results using SD is more appropriate. However, to evaluate precision of a method CV’s is more appropriate.

Question
Please provides the p values for the significant genes in Figure 5.

Response: Thanks for the reviewer’s comments and we have re-written this part according to the reviewer’s suggestion.

Question
Many typographical and grammatical errors are present in the manuscript, too
numerous to list. Manuscript needs substantial editing.

Response: Thanks for the reviewer’ valuable comments and we have revised the manuscript carefully and the revised manuscript also have been edited by a professional native-English speaker with scientific expertise.

Question

A number of studies have been published examining peripheral blood gene expression in relationship to coronary disease; it is recommended that the authors look at these manuscripts to see what has already been done in this field (PubMed IDs 19750006, 21443790, 16433769)

Response: Thanks for good suggestion and we have read them carefully. Our manuscript had been revised also.

Question

Please add label to Y axis in Figure 2, and label the peaks with corresponding gene names.

Response: Dye signal has been added to Y axis in Figure 2.

Question

Please use HUGO gene names for gene identification (e.g. SELL for L-Selectin,etc).

Response: Gene names have been checked and revised in manuscript.

IL1B, interleukin 1, beta
IL6, interleukin 6
IL8, interleukin 8
IFNG, interferon, gamma
MCP-1, CCL2 chemokine (C-C motif) ligand 2, MCP-1
VWF, von Willebrand factor
MTHFR, methylenetetrahydrofolate reductase
SELL, selectin L
TNFalpha, tumor necrosis factor alpha
MCSF, colony stimulating factor 1 (macrophage)
ICAM1, intercellular adhesion molecule 1
ID2, inhibitor of DNA binding 2, dominant negative helix-loop-helix protein
HMOX1, heme oxygenase (decycling) 1
LDLR, low density lipoprotein receptor
ACTB, actin, beta
GK, glycerol kinase

Special thanks to you for your good comments.

Reviewer #2: Tomasz Dziedzic

**Question**

The studied group is not well characterized. Were the patients with CAD symptomatic? Which criteria were used to diagnose CAD on CT? What was a distribution of vascular risk factors (hypertension, diabetes mellitus, hypercholesterolemia, smoking etc.) in patients and controls? What about drugs that could change inflammatory markers expression (aspirin, statins, ACE-I etc.)? Of note, the increased expression of inflammatory genes could be related to diseases such as diabetes or hypertension. Thus, CAD group and control group should be matched not only for age and sex, but also for major vascular risk factors. Alternatively, 2 control groups should be used: healthy subjects without vascular risk factors and persons with vascular risk factors, but without CAD.

Response: I thank for the reviewer’s helpful comments. We are very sorry for our incorrect writing the studied group. It is characterized in the revised manuscript. According the plaque density by CT, the plaque classified criteria was follows:
Noncalcified plaque showed a density<150 HU (Hounsfield Units), and calcified plaque >300 HU. The patients with CAD symptomatic or without were all recruited. Vascular risk factors include hypertension, diabetes mellitus, hypercholesterolemia, smoking were matched.

It is a good suggestion that concern the changes of drugs on inflammatory markers expression. We are very sorry for forgetting list of vascular risk factors in the first manuscript. History of major CAD risk factors, such as hypertension, diabetes mellitus, hyperlipidemia, and smoking were showed in Table 1 in the second manuscript. The drugs also be concerned and was matched in different group. The suggestion about the group is constructive. In the first step, we want to find some information between group C (noncalcified plaque, and combination group) and group A (control group without plaque). In the next step, we could analyze the vascular risk factors on the plaque. However, the number of patients evaluated was rather low, much more work need to validate in future.

**Question**

The choice of analysed genes should be justified. It seems reasonable that before planning multiplex experiments, separate PCR studies should be done to find the appropriate genes (selected from previously published data or chosen due to their role in pathogenesis of CAD) whose expression is significantly changed in blood cells of patients with CAD. It is important, because the expression of only 4 from 15 analysed genes was different between groups. Thus, it is questionable if it makes sense to measure the expression of 15 molecules.

**Response:** It is true that before planning multiplex experiments, we had selected genes according to previously published data and all selected genes might play an important role in the pathogenesis of atherosclerosis by previous reports. However, there were few reports to concern the relationship between these genes. So we have developed a new multiplex polymerase chain reaction assay to measuring them together in one reaction system and then to compare which gene play more important role related with CAD. The previously published individual data showed all the selected gene has
significant differences, There were only 4 from 15 analyzed genes had significant
different, and then an independent set of test confirmed our results.

**Question**

When one looks at Figure 5, it is clear that although there is a significant difference in
expression of 4 genes between groups, the values from CAD patients and controls are
overlapped. Thus, in clinical practice it will be difficult to differentiate persons with
and without CAD. The authors should used the cut-off point (for example values
below 2 standard deviation of mean obtained in control group) to assess if the results
of test correctly classify participants into 2 groups. Does the combination of 4 markers
differ better discriminate patients from controls than single markers?

**Response:** I thank for the reviewer’ suggestions. However, the gene expression level
is not normal distributed. Data are presented as median (interquartile range) and
cannot be presented as mean and standard deviation. The combination of 4 markers by
receiver operating characteristic curve (ROC) analysis better discriminate patients
from controls than single marker. It was shown in below Table.

<table>
<thead>
<tr>
<th>gene</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1B</td>
<td>85.5</td>
<td>50.0</td>
<td>0.639</td>
</tr>
<tr>
<td>IL6</td>
<td>43.5</td>
<td>91.7</td>
<td>0.623</td>
</tr>
<tr>
<td>IL8</td>
<td>47.8</td>
<td>80.6</td>
<td>0.642</td>
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<tr>
<td>MCP-1</td>
<td>49.3</td>
<td>80.6</td>
<td>0.624</td>
</tr>
<tr>
<td>IL1B+IL6+IL8 +MCP-1</td>
<td>50.7</td>
<td>77.8</td>
<td>0.669</td>
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</table>