Author’s response to reviews

Title: Genetic variations on chromosome 15q14-22.1 for type 2 diabetes candidate region in the Japanese population

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Author’s response to reviews: see over
February 9, 2008

Prof. Melissa Norton, MD
Editor-in-Chief

BMC Medical Genetics

Dear Prof. Melissa Norton, MD:

We sincerely appreciate your e-mail letter of January 5, 2008 concerning our revised manuscript (MS: 1340804552153919) with Reviewer 1’s constructive comments and instruction by Dr. Chrissie Kouremenou, Senior Assistant Editor. These comments have been very helpful in the re-revision of our manuscript. We thoroughly re-revised our manuscript and responded to all of the comments from Reviewer 1 and the Editorial office, particularly those of Reviewer 1, one by one.

Please find enclosed our re-revised manuscript entitled “Lack of association of genetic variation in chromosome region 15q14-22.1 with type 2 diabetes in a Japanese population” and a complete list of our responses to Reviewer 1’s comments.

We sincerely hope that our re-revised manuscript now meets the high scientific standard of your journal, and that you will now consider it suitable for publication in BMC Medical Genetics. This manuscript has not been previously published elsewhere, nor is under consideration for publication elsewhere. All authors approved of the re-revised manuscript submitted for publication.

Respectfully yours,

Mitsuo Itakura, M.D., Ph.D.
Professor and Director
Division of Genetic Information
Institute for Genome Research
The University of Tokushima
Response to Reviewer 1’s comments (1238830750174476_comment.pdf)

We appreciate your thoughtful comments. They have been very helpful in re-revising the manuscript. The manuscript has been thoroughly re-revised in response to all of your comments and, thus, contains substantial changes in the main text, Tables, Figures, and Additional files. The re-revised portions are in red font for your attention. Please find below our detailed responses to each of your comments.

General comment

This is the revised manuscript reporting dense SNP mapping on chromosome 15q14-15q22.1. The manuscript is revised, but many of the issues raised in the initial review are not well addressed. The authors now state that the methods used in this study resulted from starting the study apparently before HapMap data were available. They use a two stage design in a reasonable sample of 1794 individuals, but they now concur that power for expected effect size was in fact fairly low. This alone does not diminish the importance of a report with a dense map, but combined with essentially negative data, a SNP map that appears not to capture the full variation in the region (particularly intronic and intergenic variation), and a long, redundant, and not well written manuscript, this Reviewer enthusiasm for the work is limited. If the authors believe that this region is important, surely they should place additional SNPs in this region based on Japanese and Chinese HapMap samples and perform the study correctly. The Revision has added considerable text, but remains highly redundant.

Response-General comment

Although we respond to each point in detail in the following sections, our response to the General comment is as follows: We agree with your point that our set of SNP markers should be compared with HapMap JPT SNP data (currently the most advanced dataset with LD information) to assess how much of the full variation in the region was captured by our set of SNP markers. We thus comprehensively compared our markers with those in the HapMap JPT database (newly prepared Figure 1), and described the even representation of HapMap JPT tag SNPs by our SNPs, although the degree of representation is limited, as explained in our response to Comment-3. In addition, we evaluate the SNPs from HapMap database using
Tagger program (http://www.broad.mit.edu/mpg/tagger/), which produced a summary of tag SNPs of 30% to capture all variants of target region in the HapMap data. We added the URL for the Tagger program (Reference no. 30) to the re-revised References [line 17 on page 21].

Future studies should place additional SNPs in this region based on Japanese HapMap samples. However, we insist that the value of our study is that it is the first region-wide association study performed on Chromosome 15q14-22.1 in the Japanese population. We emphasize the importance of Chromosome 15q14-22.1 as a candidate region of T2D based on multiple linkage scan data in Japanese subjects as explained in the Background and Discussion sections.

Genetic variants that alter the risk for complex diseases are expected to have only a small effect on disease outcome within the population. For example, six recent genome-wide association study (GWAS) independently provided evidence of association between type 2 diabetes (T2D) and genetic variation (a key SNP, rs7903146) of TCF7L2. However, the risk allele frequency of rs7903146 was very low in Japanese subjects compared with Caucasians [Diabetologia 2007, vol.50:747-51]. Population dependent differences in allele frequency have also been reported for K121Q in ENPP1 [J Hum Genet 2006, vol.51:559-66] and Pro12Ala (rs1801282) in PPARG [Diabetes 2001, vol.50:891-94]. In the absence of convincing association data on chromosome 15q, it made sense to analyze the association. Even though our association test suggested a lack of susceptibility variants, our data on chromosome 15q could help uncover insights on the susceptibility variant(s) for T2D on chromosome 15q14-22 in the Japanese population.

We drastically shortened and edited manuscript by eliminating redundancy and many of the additional files.

**Major Compulsory Revisions**

[Comment-1]

As discussed above, this manuscript is 20 pages with multiple additional files, many figures and tables, all for essentially a negative study. The authors have provided no reason that UBR1 should be considered a diabetes candidate gene indeed, they spend no effort even telling the reader what it does. SNP2140 is only weakly associated, and in the two stages design barely meets 0.05 cutoffs at either stage. Considering 1317 SNPs in Stage 1 (i.e., over 200 expected to be significant
by chance depending on LD), and 112 in Stage 2 (20 expected by chance), the findings in this paper would properly be interpreted as negative. Using the two samples together, the p value is a bit more impressive at 0.004 and OR of 1.26, but with so little power to detect genes with this effect size, the interpretation is impossible. Apparently no other SNP in $UBR1$ was associated.

Response-1

1) We interpreted our results as a negative study based on the fact that none of the 1,317 SNPs exhibited a strong effect on the risk of T2D in the target region. As you mentioned, the power can better be assessed based on the power calculation by incorporating sample size, MAF, odds ratio, and a type 1 error, and power was fairly low in this sample set. However, we have confirmed the significant association of E23K allele in $KCNJ11$ [J Hum Genet, 2007, vol.52:781-93] and rs7903146 in $TCF7L2$ [this finding is under preparation as is presented here as confidential data] with T2D by applying a similar study design with our samples. These results do not negate the value of our negative results reported here, but rather encourage us to conduct replication studies with a larger sampling of independent Japanese subjects using more marker tag SNPs, such as those of the HapMap JPT SNPs in the current study.

2) We added information on the $UBR1$ gene in the Discussion section as follows:

[re-revised Discussion, lines 11-18 on page 14]

$UBR1$ encodes an E3 ubiquitin ligase of the N-end rule pathway, a conserved proteolytic pathway of the ubiquitin system whose substrates include proteins with destabilizing N-terminal residues. Recent data suggest that in Johanson-Blizzard syndrome (OMIM 243800), the pancreas exhibits pancreatic exocrine insufficiency and does not express $UBR1$ [35]. $Ubr1$ knockout mice exhibit decreased body weight or adipose tissue and an exocrine pancreatic insufficiency [35, 36]. In addition, $UBR1$ mRNA is elevated in the atrophic muscles of diabetic rats [37]. At present, however, the pathophysiological mechanism by which the $UBR1$ influences T2D is unknown.

[Comment-2]
This manuscript is at least 50% too long. It should be shortened considerably given the negative study. Analyses such as EHH and REHH seem to add little.

**Response-2**

According to your clear advice, we edited re-revised manuscript as follows:

1) We shortened the length of the main text from 4,977 words to 3,114 words (excluding references).

2) We eliminated the description and related Table, Figure, and Additional files detailing EHH and REHH, comparison of our SNP markers with JSNP, LD block structure of the 21.4 Mb region, and comparison of the LD block structure around *UBR1* using HapMap data.

3) We eliminated the redundant descriptions.

4) The re-revised manuscript contains three Tables, three Figures, and two Additional files. For the two Additional files, we responded in Response-15 to Major Compulsory Revisions. We changed the Tables and Figures as follows:

<table>
<thead>
<tr>
<th>revised text</th>
<th>re-revised text</th>
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<tbody>
<tr>
<td>Additional file 1</td>
<td>Table 1 (insertion in text)</td>
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<tr>
<td>Table 1</td>
<td>Table 2</td>
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<td>Table 2</td>
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<td></td>
<td>Newly created Table 3</td>
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<tr>
<td>Figure 1</td>
<td>Newly created Figure 1</td>
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<td>Figure 2</td>
<td>Figure 2</td>
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<td>Figure 3</td>
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[Comment-3]

The authors still confused this reader on where the SNPs for this study came from. The proper comparison is not JSNP, which has very limited utility, but HapMap, which is more comprehensive and provides the best data today on what total genetic variation is present. All the readers need to know is how much of the variation in HapMap was captured using the current SNP map of 1,300 SNPs as proxies and at different values of r2 (0.7, 0.8, 0.9 for example). If most intergenic SNPs were not captured, the authors may well have missed the important variants and latched onto a marginally significant one instead.
Response-3a

We explained the origin of the SNPs used in this study in the Methods section as follows:

[re-revised Methods; lines 2-8 on page 7]

At the onset of this study, there was no sufficient SNPs database, such as HapMap. Thus, we constructed a database of common Japanese SNPs based on the genotyping results of 45 unrelated Japanese control subjects (23 males and 22 females) in a whole genome in collaboration with Applied Biosystems (ABI) [18]. Out of 70,099 common (MAF >0.15) gene-centric and intergenic SNPs made available by this project, we provisionally selected 1,450 evenly-spaced SNPs on chromosome 15q between two sequence-tagged site (STS) markers of D15S118 and D15S117.

Response-3b

To explain “how much genetic variation in the target region was captured based on HapMap JPT data”, we examined the amount of genetic variation captured by our SNPs against HapMap JPT data. We explained the comparison using a newly created Figure 1 and the text as follows:

[re-revised Results; line 19 on page 10 - line 8 on page 11].

Captured genetic variation relative to HapMap JPT SNPs in the target region

Coverage of the 1,317 SNPs was more limited than that with 29,728 SNPs of HapMap JPT in the 21.8 Mb region: 1,022 SNPs (78%) of the 1,317 SNPs were present, while only 295 SNPs (22%) were not present in the 29,728 HapMap JPT (Figure 1), although they were registered in the public database [32] without information on allele frequency in the Japanese population. Figure 1 shows only one gap in 43.6-43.9 Mb in this study, and the presence of a rare density of SNPs (< 10) was observed, particularly in the intergenic region and between 42.1-44.8 Mb.

This approach is more efficient if tag SNPs based on the HapMap JPT r^2 structure are used to find the susceptibility variant [28]. We estimated the captured tag SNPs as proxies, meaning that the SNP showed a strong correlation with one or more SNPs at r^2 >0.7, 0.8, or 0.9. The density of most tag SNPs was <10 SNPs per 300-kb bin (Figure 1). Taken together, the coverage of common SNPs variation by our chosen SNP set was rather balanced in both gene-centric and intergenic regions, and the average number of common tag SNPs in this study was 30% of those in the HapMap JPT (r^2 > 0.8).
The entire discussion of LD block structure seems superfluous. These data can be obtained from HapMap, but pages are spent providing methods and data. This reviewer recommends that this be removed.

**Response-4**
We eliminated the description of LD block structure estimated by the genotyping data from 360 controls and 372 cases on chromosome 15q14-22.1 (21.8 Mb) in the Methods, Results, and Discussion sections.

The EHH and REHH sections remain impossible to understand. As this seems to add little, this Reviewer recommends that these be removed.

**Response-5**
This comment is the same as the second point in Comment-2 in Major Compulsory Revisions. LD block size and haplotype specific decay of LD pattern between populations is interpreted as presenting the population-specific selection. Because we did not find the evidence for selection in this study, we eliminated the EHH and REHH sections from the Methods, Results, and Discussion sections.

Please remove the paragraph on page 11 comparing the SNPs to JSNP. The proper comparison is to HapMap, which is not gene centered or biased. HapMap will incorporate JSNP. The new sections add nothing to the manuscript, except for information about very large gaps of 43 Mb with no SNPs.

**Response-6**
This comment is partly the same as the second point in Comment-3 in Major Compulsory Revisions. We eliminated the paragraph “Comparing of genetic variation in the target region with JSNP,” except for information about gap between 43.6-43.9 Mb, because JSNP is not a standard. We examined the amount of genetic variation
captured by our 1,317 SNPs in the HapMap JPT database in the Results section and added a newly created Figure 1.

[Comment-7]
Delete the paragraph on LD block structure on page 13; this adds very little useful data. Using a gene centered SNP map to conclude that the results provided a considerable coverage is simply flawed logic. The correct standard is HapMap, which is not gene centered. Gene centered maps can be expected to show stronger block structure

Response-7
This comment is the same as the second point in Comment-4 in Major Compulsory Revisions. We eliminated the paragraph on LD block structure on chromosome 15q14-22.1 in 21.8 Mb.

[Comment-8]
Please provide the justification for the studies on Page 13, bottom, and page 14. If UBR1 is not a strong candidate, and the association for SNP2140 is marginal, why choose additional SNPs? Why choose only these 7? Again, too much is made of block structure and LDU; the association results are never provided, which is the point of this paper. How was UBR1 resequenced did you sequence only exons? This strategy remains very unclear.

Response-8a (Reason for additional SNP typing)
SNP2140 was weakly associated with T2D in all association tests, albeit with nominal $p$ values. We could not completely rule out the possibility of genetic contributions of SNP2140 to T2D only by the single locus association test.

Haplotype-based association tests assessed as a multi-locus test can be more sensitive for the detection of association than assessment by a single-locus association test. Therefore, we performed the haplotype-based association test. As it is important to strictly confirm LD structure when performing haplotype-based association tests, we first searched for additional common variants around the putative LD block boundaries with the available database. We validated them by sequencing 32 samples or with the validated TaqMan SNPs probe from ABI. Out of
nine additional SNPs, seven TaqMan SNP genotyping assays were successfully
developed to confirm the strict LD structure. We added the explanation in the Results
section [line 20 on page 12 - line 11 on page 13].

In addition, many studies suggest the evidences that deficiency of UBR1 causes
vol.21:8007-21], as described in the Discussion section. Therefore, we conducted a
mutation analysis in the UBR1 gene.

**Response-8b** (method for re-sequencing the UBR1 gene)

The 47 coding exons, relevant intron-exon boundaries, and 5’ and 3’ UTR of
UBR1 gene were screened for putative novel Japanese variants by direct
sequencing the genomic DNA of 48 individuals (24 cases and 24 controls). We
described the method of sequencing in the section “Searching for additional or novel
SNPs within the UBR1 gene” in the Methods section [lines 17-19 on page 8]. In
addition, we eliminated the description of LD block structure and LDU, and explained
the results of association test for an additional seven SNPs [lines 2-4 on page 13]
and the results of identification of new SNPs in the coding exons in the Results
section [lines 12-18 on page 12].

[Comment-9]

This reviewer does not understand where 7 tag SNPs came from. Only 8 SNPs
were typed! If LD was strong, those 8 SNPs could surely be covered with fewer
than 7 tags. This section is simply impossible to understand as written. No reader
is realistically going to refer to 7 additional files with a long paper. Please restrict
the data to what is important for readers, and put those data in the paper. The
investigators need to seriously edit this manuscript.

**Response-9a**

We first, underscore that eight is the number of haplotypes capturing >95% of
the target block, and does not represent eight SNPs.

We described in **Response-8a**, that a multi-locus haplotype-based association
test can be more sensitive in detecting association than assessment by a single-
locus association test. Thus, although the association of SNP2140 is marginal in this
Japanese population, we thought that there was a good reason to perform this test.
We investigated the LD structure with the pairwise $|D'|$ values of the region that includes the $UBR1$ gene. Using the definition of $|D'| > 0.95$, we identified a large LD block consisting of 38 SNPs including seven additional SNPs spanning 355 kb across the $UBR1$ gene. Thus, to infer haplotype, we selected seven haplotype-tagging SNPs (htSNPs) from 38 SNPs that captured $> 95\%$ of haplotype frequencies comprising the respective block in all samples. This explanation has been added to the Results section [lines 5-11 on page 13]. In addition, we described the correction for multiple testing as “corrected for 1,317 SNPs multiplied by two + eight haplotypes”.

Response-9c (edit 7 additional files)

This comment is the same as the Comment-15 in Major Compulsory Revisions. We eliminated five Additional files from the manuscript as detailed in Response-15.

[Comment-10]

Remove the paragraph a comparison of the landmark LD block. This information has no bearing on the role of $UBR1$ in diabetes. Similarly the next paragraph in this Reviewer’s opinion adds nothing.

Response-10

This comment is the same as that in Comment-2 and 5 in Major Compulsory Revisions. We eliminated the paragraph “Comparison of the landmark LD block with other populations using HapMap data” and the next new paragraph “Application of EHH and REHH analyses in the landmark LD block” from the text.

[Comment-11]

Page 19, the HapMap data should have at least been used in the region of $UBR1$; not doing so leaves the paper with limited interest.

Response-11

Because of Reviewer 1’s comment “Page 19, the HapMap data should have at least been used in the region of $UBR1$” directed at the LD block and EHH and REHH, these descriptions were eliminated from the text and the comment is no longer applicable.
The new paragraph comparing LD between Japanese and YRI is of no real interest. Sufficient publications are available showing marked differences among populations, particularly YRI vs CEPH or Asian samples. This really contributes little but length and is recommended to be removed.

**Response-12**

This comment is the same as that in Comment-2, 5 and 10 in Major Compulsory Revisions. We eliminated the paragraph “Comparing LD between Japanese and other population, especially from YRI (the paragraph describing EHH or REHH)” from the text.

Table 2 can probably be removed; it provides little usable data. What matters is how well the total genetic variation was captured in this study; JSNP is not the standard, and simply counting SNPs is not helpful. Similarly Figure 1 adds very little. A more useful figure would be SNP density by distance, showing gaps.

**Response-13**

This comment is the same as Comment-3 and 6 in Major Compulsory Revisions. We eliminated revised Table 2 and Figure 1 presenting the comparison of SNP distribution between this study and JSNP from the text. Our response to captured genetic variation was summarized in Response-3 under Major Compulsory Revisions.

Did the authors look at Stage 1 + Stage 2 for the stronger SNPs in Stage 1? Did they only look at the arbitrary 0.05 cutoffs for both stages separately? Given the poor power of Stage 1, this might be an error. Please present the combined data for all SNPs with Stage 1 \( p < 0.01 \).

**Response-14**

As you requested, we have presented the combined \( p \) values for all Stage 1 SNPs with \( p \leq 0.01 \) for either the allelic or genotypic test in Additional file 1 including...
results of genotype frequencies. We estimated the \( p \) values of 28 SNPs in Stage 1 + Stage 2 combined samples for the allelic or genotypic test with significant associations \( (p \leq 0.01) \) in Stage 1. The results of association for the top 20 Stage 1-associations using the Armitage trend test (http://ijg.gsf.de/cgi-bin/hw/hwal.pl) are shown in newly created Table 3. As shown in Table 3, three SNPs (SNP3347: rs1386166, SNP3096: rs156787, and SNP2072: rs936216) showed a smaller \( p \) value after allelic testing than the landmark SNP2140 based on the combined \( p \) values with significant associations.

Many reports have indicated that joint analysis is more efficient and powerful than the standard two stage strategy, which tests statistics for the second stage data alone (Nat Genet 2006, vol.38:209-13). However, none of the above three SNPs showed significant association in Stage 2, although sample size and power to detect susceptibility SNPs was larger than in Stage 1. We believe that the SNP with replicated significance in both Stage 1 and Stage 2 is regarded as the more likely SNP for T2D association. Unfortunately, none of 112 SNPs reached the statistical confidence level in our sample population. All told, we did not regard these three as putative T2D-associated SNPs. To confirm our data, it will be necessary to test association using a larger number of independent samples. We cited this issue in the Results section [lines 3-11 on page 12].

[Comment-15]

8 additional files are excessive. This reviewer and all but a few readers will not have time to open and examine each file. The vast majority of these are not essential. The linkage region should be shown in the manuscript, and the manuscript should show a table of the top 20 associations from Stage 1 using a well accepted test (Armitage Trend is a good choice). They can then provide Additional File 2, but clearly only 112 actually had second stage and combined data. The current submission is overly confusing and impossible to read.

**Response-15 a**

We have eliminated five Additional files, and inserted an Additional file 1, which presents a summary of the linkage studies as Table 1, as follows:

<table>
<thead>
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<th>revised manuscript</th>
<th>re-revised manuscript</th>
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<tbody>
<tr>
<td>Additional file 1</td>
<td>Table 1 in text</td>
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</table>
Response-15 b

We created a new Table 3 that shows the results of association testing for the top 20 Stage 1-associations using the Armitage trend test in the text [Nature Rev Genet 2006, vol.7: 781-91]. In addition, following your suggestion, we added combined p value data for the 28 SNPs exhibiting significant p values (p ≤0.01) in Stage 1.

Minor Essential Revisions

[Comment-1]
Change the title to Genetic variation (not plural).

Response-1

Based on your and the Editorial office’s suggestions, we changed the title of the re-revised manuscript to “Lack of association of genetic variation in chromosome region 15q14-22.1 with type 2 diabetes in a Japanese population”.

[Comment-2]
Please add sufficient description of the two stage design (number of subjects in each stage) in the abstract.

Response-2

We explained the two stage design by describing the number of subjects in each stage in the re-revised Abstract [lines 8-13 on page 3].

[Comment-3]
The grammar throughout is problematic. For example, Background have 
replicated confirmed 11 genes makes no sense; do the authors mean have 
confirmed 11 genes with replicated associations with T2DM in Caucasians? Similar 
problems are present throughout the manuscript, and require careful editing of the 
English. On page 5, last sentence, change was closely examined to be closely 
examined. On Page 8, please add 372 cases and 360 controls if this is appropriate, 
and change false positive to false positive rate.

Response-3

We contacted a professional editing service and had the re-revised manuscript 
comprehensively edited by a native English speaker.

Response-4

Though there are many reports in which this software was used, there is not an 
original publication. We added the URL for the SNPAlalyze program (Reference no. 
27) to the re-revised References [line 13 on page 21]

Response-5

We thoroughly clarified the description of all p values by in Stage 1, Stage 2, and 
for the combined samples in the manuscript. In particular, a p value of 0.0043 for the 
combined samples is described in the Discussion section [line 10 on page 14].

Response-6

No real need for Figure 3D; the text is sufficient to understand the power 
limitations.
We eliminated Figure 3D in the text.

[Comment-7]  
Page 18, paragraph “The density of SNP markers…” is unclear; this needs to be reworded, and this entire section is highly redundant.

Response-7  
According to your suggestion, we have eliminated the redundant paragraph “The density of SNP markers…” in the text.

[Comment-8]  
Page 19, tag SNPs were not available.

Response-8  
We corrected “Even though tag SNPs was not available…” to “Even though tag SNPs were not available…” in the Discussion section [lines 21-22 on page 15].

[Comment-9]  
Page 29, the figure legend is confusing. The manuscript states 1794 subjects, but here the authors list 372 Stage 1, 532 Stage 2, and 904 Stage 1 + Stage 2 combined samples. Please clarify. Was power based only on cases?

Response-9  
The PS program used in this study has two output sections of calculated power and sample size. To calculate the sample size with a specific power, we input the ratio of control to case, the type 1 error probability for a two sided test, and the odds ratio. Thus, calculation is based on the number of cases and controls. The confusing description in the figure legend was corrected [lines 3-6 on page 24].
Response to the Editorial office comments

We sincerely appreciate your kind comments. We have re-revised our manuscript in response to Associate Editor's comments.

Comments

Although a shorter manuscript will be easier for readers, we would not insist that you shorten the manuscript by 50%, as the reviewer asks.

Please revise the title from "Genetic variations on chromosome 15q14-22.1 for type 2 diabetes candidate region in the Japanese population" to "Lack of association of genetic variation in chromosome region 15q14-22.1 with Type 2 diabetes in a Japanese population".

Responses-1

We have changed the title of the re-revised manuscript to "Lack of association of genetic variation in chromosome region 15q14-22.1 with Type 2 diabetes in a Japanese population".

Responses-2

Following the recommendation of the Editorial office, we shortened the length of the main text from 4,917 words to 3,114 words (excluding references).