Author's response to reviews

Title: SNP55, a new functional polymorphism of MDM2-P2 promoter, contributes to allele-specific expression of MDM2 in endometrial cancers

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Author's response to reviews: see over
Dear Dr. Sands

We are pleased to submit the revised version of our manuscript entitled “SNP55, a new functional polymorphism of MDM2-P2 promoter, contributes to allele-specific expression of MDM2 in endometrial cancers”.

We have revised the manuscript according to the comments from editors and reviewers.

We attach here our revised manuscript, as well as a point-by-point response to the reviewer’s comments.

We feel that the comments have allowed us to improve the paper and hope you convey our gratitude to the reviewers. We now hope that our paper will be suitable for publication in BMC Medical Genetics and look forward to hearing from you concerning your editorial decision.

Yours sincerely,

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Point-by-point response

Referee 1:

Major Compulsory Revisions 1)

We started this research to investigate whether the ASE actually exists in vivo, and conducted the PLACE-SSCP analysis. It revealed that the MDM2-ASE actually exists in endometrial cancer tissue samples and this expression difference seemed to originate from the MDM2-P2 promoter as described in the manuscript. Then, we attempted to elucidate the origin of the MDM2-ASE. The first candidate was SNP309, because there are many reports which show that it SNP309 causes the MDM2-ASE. We hypothesized that the status of SNP309 (G or T) was heterozygous in our ASE-positive samples if it caused the ASE-phenomenon observed. Unexpectedly, however, one of
our ASE-positive samples showed homozygous (T/T) in SNP309. Next, we planned to investigate genomic sequences of total MDM2-P2 promoter region to search new SNP candidates. Because we found that the region around MDM2-P2 promoter is GC-rich, we expanded the size of amplicon up to 1000base pairs including total MDM2-P2 promoter region to amplify the target region precisely. The sequence data of the ASE-positive endometrial cancer tissue samples was analyzed using the NCBI SNP database to search new candidates which can cause the MDM2-ASE. It revealed that the SNP55 was the only candidate, that is, only SNP55 was heterozygous in our sample investigated.

We think that SNP309 and SNP55 may be in LD, because the genotype frequencies of them are quite different in the NCBI SNP database.

We could not make a definite conclusion about the haplotype structure between the SNP309 and the SNP55 because we analyzed only small number of samples in this study.

We would like to apologize for the difficulty in the SNP representation. However, we are afraid that it makes the readers more confused if the SNP representation is changed to describing both rs## and SNP## side by side for the whole manuscript. Instead, we added both information (rs## and SNP##) to Keywords section and to the first words where it comes out to the manuscript (Background section, SNP55 : p7, line5, SNP309 : p6, line20).

**Major Compulsory Revisions 2)**

At the beginning of this study, we attempted to confirm that the SNP309 actually caused MDM2-ASE in vivo. For this purpose, we conducted the PLACE-SSCP analysis in human endometrial samples and found a new functional polymorphism, SNP55. Our point is not that the MDM2-ASE is observed only in endometrial cancers and it is possible that the same phenomenon is observed in other tissue samples, although we never tested them.

To our knowledge, the eQTL was found without any extraordinary stimuli. In contrast, our result indicated that not only the status of SNP55 but also subcellular localization and dimerization status of NFkB p50 affect the MDM2-ASE. It is thought that some special stimuli might be necessary for NFkB p50 to form homodimer and translocate to the nucleus. Accordingly, we think that it was difficult for the eQTL to find SNP55 and it is quite reasonable that there is no information of SNP55 in other reports using eQTL.

**Minor Essential Revisions 1)**

Before the first submission, this manuscript was carefully reviewed by experienced editor whose first language is English and who specializes in editing papers written by scientists whose native language is not English in advance. In addition, the other referee commented that the quality of written English was acceptable. Please tell me why you felt it had many grammatical problems in detail.

**Minor Essential Revisions 2)**
Before the first submission, this manuscript was carefully reviewed by experienced editor whose first language is English and who specializes in editing papers written by scientists whose native language is not English in advance. In addition, the other referee commented that the quality of written English was acceptable. Please tell me why you felt necessity of extensive editing in detail.

Referee 2:

**Major Point 1)**

We totally agree with the referee’s opinion and would like to change the title from “SNP55, a new functional polymorphism of MDM2-P2 promoter affected by NFkB p50 in endometrial cancers” to “SNP55, a new functional polymorphism of MDM2-P2 promoter, contributes to allele-specific expression of MDM2 in endometrial cancers”.

**Major Point 2)**

We confirmed the effect of SNP55 on the binding affinity of transcription factors in figure 5. Next, we attempted a luciferase assay to investigate the effect of SNP55 on transcriptional activity of the MDM2-P2 promoter. We constructed two reporter plasmids, both of which contained the MDM2-P2 promoter region with different SNP55 status in the upstream of the luciferase gene. We planned to perform a luciferase assay to confirm the effect of the SNP55 on the MDM2-P2 promoter activation under the presence of both transcription factors, Sp1 and NFkB p50. The transcription factor Sp1 localizes in the nucleus and NFkB p50 localized in the cytoplasm in normal condition. At first, we planned to establish stable cell lines of each reporter plasmids described above, and to stimulate cells for endogenous NFkB p50 to form homodimer and translocate to the nucleus. However, there were few reports describing what triggers the NFkB p50 to form homodimer and translocate to the nucleus. So we constructed the expression vectors of Sp1 and NFkB p50, respectively, and co-transfected with the reporter plasmids transiently. Almost all human cell lines express Sp1. It is possible that endogenous Sp1 might affect the result of the experiment if using cell line derived from human. The COS-1 cells do not express the human Sp1 and are used as a host cell line in the transfection experiment of the human Sp1 in elsewhere.

(Reference)

**Major Point 3)**

We totally agree with your indication. The number of samples analyzed in this paper was too small to assert that the SNP55 polymorphism
was not associated with the endometrial cancer risk.
So, we would like to change the two sentences in this paper.

(1) Abstract-Result

(before : p3, line22-23)
this result suggest that SNP55 alone does not affect endometrial cancer risk.

(after : p3 line22-23)
this result might suggest that SNP55 alone does not affect endometrial cancer risk.

(2) Discussion

(before : p20, line14-15)
This result has shown that SNP55 polymorphism was not associated with endometrial cancer risk.

(after : p20, line14-17)
This result might have shown that SNP55 polymorphism was not associated with endometrial cancers. However we could analyze only small number of samples in this paper, and could not make a definite conclusion.

Minor Point 1)

We added a new sentence mentioned it in the Methods: Statistical analysis region

(before : p13, line8-9)
A $P$-value of $<0.05$ was considered statistically significant.

(after : p13, line8-10)
The $P$-values given were two-sided and a $P$-value of $<0.05$ was considered statistically significant.