Author’s response to reviews

Title: Genetic Polymorphisms in Bone Morphogenetic Protein Receptor Type IA Gene predisposes individuals to Ossification of the Posterior Longitudinal Ligament of the Cervical Spine via the Smad signaling pathway

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Version: 3 Date: 27 Jan 2018

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BMSD-D-17-00981R2

Title: Genetic Polymorphisms in Bone Morphogenetic Protein Receptor Type IA Gene predisposes individuals to Ossification of the Posterior Longitudinal Ligament of the Cervical Spine via the Smad signaling pathway

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BMC Musculoskeletal Disorders

Dear Editor Dr. Anne Gingery,

Above all, we sincerely thank again members of editorial review board for your hard working and giving us lots of valuable revision advices. We appreciate your response and overall positive initial feedback, and made modifications to improve the manuscript. After carefully reviewing the comments made by the reviewers, my manuscript has been strictly revised according to reviewers’ comments. A list of changes for each point raised is appended below.

If the revised manuscript has inappropriate according to reviewers' comments, we are willing to make further revision.

We hope that you will find the revised paper suitable for publication, and we look forward to contributing to your journal. Please do not hesitate to contact us with other questions or concerns regarding the manuscript.

Best regards,
A list of changes for each point raised is as follows:

Chikenji Takako (Reviewer 1):

Major Comments

The authors performed ANOVA followed by Bonferroni post hoc test in their statistical analysis instead of LSD posthoc multiple comparison as I mentioned before.

In their discussion, they have addressed "the phosphorylated R-Smads recruit the common mediator Smad (Co-Smad4) and forms hetero-oligometric complexes", however, in their results, Smad4 was not different in any cells, only the p-Smad1/5/8 was increased in pcDNA3.1/BMPR-IA (MT -349C>T) and (MT -349C>T and 4A>C) cells compared as WT cells. How do we understand the results? They should discuss these results theoretically in their manuscript. If they would address "Smad signaling pathway may play important roles in the pathological process of OPLL induced by SNPs in BMPR-IA gene" in their conclusion, their results would not be theoretical.

Response: Above all, we thank you for reviewing my manuscript and giving me lots of valuable revision advices. Indeed, in our results, the common-mediator Smad4 (Co-Smad4) protein was not different in any cells, only the phosphorylated receptor-activated Smads (R-Smads, Smads 1, 5 and 8) proteins were increased in pcDNA3.1/BMPR-IA (MT -349C>T) and (MT -349C>T and 4A>C) cells compared as WT cells. Signal transduction studies have revealed that Smads 1, 5 and 8 are the immediate downstream molecules of BMP receptors and play a central role in BMP signal transduction. R-Smads are the only Smads that have an SSXS motif in their C-terminal region. Smads 1, 5 and 8 transiently and directly interact with activated type I BMP receptors, which phosphorylate the C-terminal SSXS motif of Smad in a ligand-dependent manner. After release from the receptor, the phosphorylated Smads 1, 5 and 8 proteins form hetero-oligomeric complexes with the common-mediator protein Smad4, which acts as a shared partner. The R-Smads (Smads 1, 5 and 8) and Co-Smads (Smad4) complexes translocate into the nucleus to regulate the transcription of specific target genes with other transcription factors. In the nucleus, Smads 1 and 5 directly bind to DNA and interact with sequence-specific DNA binding proteins for the formation of a stable DNA-binding complex. Our present results demonstrate that that the -349C>T polymorphism of BMPR-IA gene is positively associated with the phosphorylation of Smad1/5/8 expression levels. Taken together with present other experiment data, we propose that the -349C>T polymorphism of the BMPR-IA gene may plays an important role in mediating susceptibility to OPLL via Smad signaling pathway. However, no significant differences were
observed in the levels of Co-Smad4 protein among the experimental groups. These results indicate that the 4A>C and -349C>T polymorphisms of BMPR-IA gene do not affect genetic predisposition to OPLL that is mediated through the increased levels of the Co-Smad4 protein. In addition, we found that the protein levels of phosphorylated Smad1/5/8 were not increased significantly in pcDNA3.1/BMPR-IA (MT 4A>C) vector-transfected C3H10T1/2 cells compared to the pcDNA3.1/BMPR-IA (WT) vector-transfected C3H10T1/2 cells. This result led us to hypothesize that the 4A>C polymorphism in the BMPR-IA gene may increase the susceptibility and severity of OPLL through another signaling pathway in addition to Smads.

We had rewritten and added to describe the conclusions in (conclusions section, lines 9-29, Page 15): ‘Signal transduction studies have demonstrated that the immediate downstream molecules of BMP receptors are Smad proteins, especially Smad1, 5 and 8, which play a central role in BMP signal transduction. Binding of BMP ligand to at least one type I and one type II BMP receptors results in the type II BMP receptor phosphorylating the type I receptor [29], which subsequently leads to the recruitment of the receptor-activated Smads (R-Smads, Smads 1, 5 and 8). The activation of type I BMP receptor is required for the direct interaction between type I receptor and R-Smads (Smads 1, 5 and 8). R-Smads associate directly and transiently with activated type I BMP receptors and undergo direct phosphorylation at the C-terminal SSXS motif of Smad in a ligand-dependent manner [30, 31]. R-Smads are the only Smads that have an SSXS motif in their C-terminal region. After rapid release from the type I receptor, the phosphorylated R-Smads proteins form hetero-oligomeric complexes with the common-mediator Smads (Co-Smads, Smad4), which acts as a shared partner. The R-Smads and Co-Smad complexes then translocate into the nucleus to regulate the transcription of specific target genes with other transcription factors. In the nucleus, the Smads 1 and 5 proteins exert their transcriptional activity by direct interaction with DNA and association with other DNA-binding proteins [32]. ALP, OC, and type I collagen are osteogenesis-specific protein factors in the downstream regulation of the BMP signaling pathway. Moreover, ALP is essential for the osteogenic phenotype and is critical for bone formation mediated by the regulation of the mineralization of bone matrix [33, 34].’

and in (conclusions section, line 22-, Page 16, line 12, Page 17): ‘Based on previous BMP receptor signal transduction studies, we propose that the -349C>T polymorphism of the BMPR-IA gene may play an important role in mediating susceptibility to OPLL via Smad signaling pathway. However, no significant differences were observed in the levels of Co-Smad4 protein among the experimental groups. There were no significant differences in the OC activity between pcDNA3.1/BMPRIA (WT) vector-transfected C3H10T1/2 cells and pcDNA3.1/BMPRIA (MT -349C>T, MT 4A>C, MT -349C>T and 4A>C) vector-transfected C3H10T1/2 cells. These results indicate that the -349C>T and 4A>C polymorphisms of BMPR-IA gene do not affect genetic predisposition to OPLL that is mediated through the increased levels of the Co-Smad4 protein and OC activity. In addition, we found that the protein levels of phosphorylated Smad1/5/8 and the ALP activity were not increased significantly in pcDNA3.1/BMPR-IA (MT 4A>C) vector-transfected C3H10T1/2 cells compared to the pcDNA3.1/BMPR-IA (WT) vector-transfected C3H10T1/2 cells. This result led us to hypothesize that the 4A>C polymorphism in the BMPR-IA gene may increase the susceptibility and severity of OPLL through another signaling pathway in addition to Smads. Based on previous studies, in addition to Smad signaling pathway, BMP binding to a homomeric type I or
II BMPR leads to secondary formation of heterotetrameric complexes that activates non-Smad signaling pathways such as mitogen-activated protein kinase (MAPK) family of molecules including p38 and ERK1/2.’

Marina Feigenson (Reviewer 2):

The authors answered my concerns and the manuscript can be accepted.

Response: Thank you for reviewing my manuscript and giving me lots of valuable revision advices.