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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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

Hao Wang; Wei Tao Jin; Hai Bin Li

BMC Musculoskeletal Disorders

Dear Editor Dr. Anne Gingery,

Above all, we sincerely thank 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 and the grammar has been corrected by a professional editor of English language. 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 third 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,

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

Chikenji Takako (Reviewer 1):

GENERAL COMMENTS

In this study, the authors carried out SNPs in BMPR-IA genotyping in 356 OPLL patients and 617 non-OPLL patients, and 4A>C and -349C>T polymorphisms of BMPR-IA was significantly higher in the OPLL patients than those in control group. Transfection of this BMPR-IA in the C3H10T1/2 cells were performed and phosphorylated Smad1/5/8, and Smad4 expression of the transfected cells were examined by western blotting, and alkaline phosphatase and osteocalcin activity were also examined. Although other investigations have reported the association between BMP SNPs and OPLL (Liang Yan et al. Aging Dis. 2017, doi: 10.14336/AD.2017.0201), this study have weakness in Vitro experiment and statistical analysis, which would be difficult to suggest "the Smad signaling plays a major role in the pathological process of OPLL induced by SNPs in BMPR-IA gene" as in their conclusion.

SPECIFIC COMMENTS

1. Please address sample size of all In Vitro experiment. In addition, the authors used ANOVA followed by LSD posthoc multiple comparison in their in vitro study. Fisher's LSD is restricted to 3 groups comparison. The LSD method does not control family-wise α error level. Therefore it is inappropriate for multiple comparison procedure where the control of family-wise α error level is necessary (PMID: 25984481), and the LSD method can not be applied in the case of 4 groups or more. Please reconsider the statistics methods and indicate statistical software which you used.

Response: We had added sample size (n=5) of all in Vitro experiment and the explanation of "*" in the Manuscript body. 1.(Results section, lines 3-14, Page 11):”The results demonstrated that the BMPR-IA protein levels were significantly increased in pcDNA3.1/BMPR-IA (WT, MT -349C>T, MT 4A>C, MT -349C>T and 4A>C) vector-transfected C3H10T1/2 cells compared to the control cells and empty pcDNA3.1 (+) vector-transfected cells (n=5, *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA WT cells compared with C3H10T1/2 cells, *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA WT cells compared with C3H10T1/2/pcDNA3.1 cells;
**p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2 cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT 4A>C cells compared with C3H10T1/2 cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T and 4A>C cells compared with C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T and 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells).’

2. (Results section, lines 19-25, Page 11): ‘In addition, the BMPR-IA protein levels in pcDNA3.1/BMPR-IA (MT -349C>T, MT 4A>C, MT -349C>T and 4A>C) vector-transfected C3H10T1/2 cells were higher than in pcDNA3.1/BMPR-IA (WT) vector-transfected C3H10T1/2 cells (n=5, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1/BMPRIA WT cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT 4A>C cells compared with C3H10T1/2/pcDNA3.1/BMPRIA WT cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T and 4A>C cells compared with C3H10T1/2/pcDNA3.1/BMPRIA WT cells).’

3. (Results section, lines 2-13, Page 12): ‘The phosphorylation levels of Smad1/5/8 were increased significantly in pcDNA3.1/BMPR-IA (WT, MT -349C>T, MT 4A>C, MT -349C>T and 4A>C) vector-transfected C3H10T1/2 cells compared to control C3H10T1/2 cells and empty pcDNA3.1 (+) vector-transfected C3H10T1/2 cells (n=5, *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA WT cells compared with C3H10T1/2 cells, *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA WT cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2 cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1 cells; *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT 4A>C cells compared with C3H10T1/2/pcDNA3.1/BMPRIA WT cells, *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2 cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T and 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells).’

4. (Results section, lines 16-20, Page 12): ‘In addition, the phosphorylation levels of Smad1/5/8 were significantly increased in pcDNA3.1/BMPR-IA (MT -349C>T, MT -349C>T and 4A>C) vector-transfected C3H10T1/2 cells compared to pcDNA3.1/BMPR-IA (WT) vector-transfected C3H10T1/2 cells (n=5, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1/BMPRIA WT cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1/BMPRIA WT cells).’

5. (Results section, line 23, Page 12): ‘However, no significant differences were observed in the phosphorylation levels of Smad1/5/8 between pcDNA3.1/BMPR-IA (MT 4A>C) and pcDNA3.1/BMPR-IA (WT) vector-transfected cells (n=5).’

6. (Results section, line 24, Page 12): ‘The Smad4 protein levels also did not differ significantly among the experimental groups (n=5).’
The ALP activity was increased in pcDNA3.1/BMPR-IA (WT 23.67±0.42 U/gprot, MT -349C>T 30.56±0.44 U/gprot, MT 4A>C 23.84±0.42 U/gprot, MT -349C>T and 4A>C 26.91±0.41 U/gprot) vector-transfected C3H10T1/2 cells compared to control C3H10T1/2 cells (14.74±0.46 U/gprot) and empty pcDNA3.1 (+) vector-transfected C3H10T1/2 cells (15.08±0.51 U/gprot) (n=5). ALP activity was significantly higher in pcDNA3.1/BMPR-IA (MT -349C>T 30.56±0.44 U/gprot, MT -349C>T and 4A>C 26.91±0.41 U/gprot) vectors-transfected C3H10T1/2 cells compared to pcDNA3.1/BMPR-IA (WT 23.67±0.42 U/gprot) vector-transfected C3H10T1/2 cells (n=5). However, no significant differences were observed in the ALP activity between pcDNA3.1/BMPR-IA (MT 4A>C 23.84±0.42 U/gprot) and pcDNA3.1/BMPR-IA (WT 23.67±0.42 U/gprot) vector-transfected C3H10T1/2 cells (n=5). In addition, the OC activity increased in pcDNA3.1/BMPRIA (WT 1.01±0.10 ng/mL, MT -349C>T 1.04±0.12 ng/mL, MT 4A>C 1.02±0.09 ng/mL, MT -349C>T and 4A>C 1.03±0.13 ng/mL) vector-transfected C3H10T1/2 cells compared to the control C3H10T1/2 (0.72±0.11 ng/mL) cells and empty pcDNA3.1 (+) (0.79±0.12 ng/mL) vector-transfected C3H10T1/2 cells (n=5). In addition, no significant differences in the OC activity were observed between pcDNA3.1/BMPRIA (WT 1.01±0.10 ng/mL) vector-transfected C3H10T1/2 cells and pcDNA3.1/BMPRIA (MT -349C>T 1.04±0.12 ng/mL, MT 4A>C 1.02±0.09 ng/mL, MT -349C>T and 4A>C 1.03±0.13 ng/mL) vector-transfected C3H10T1/2 cells (n=5).

In addition, the authors used ANOVA followed by LSD post hoc multiple comparison in their in vitro study. Fisher's LSD is restricted to 3 groups comparison. The LSD method does not control family-wise α error level. Therefore it is inappropriate for multiple comparison procedure where the control of family-wise α error level is necessary (PMID: 25984481), and the LSD method can not be applied in the case of 4 groups or more. Please reconsider the statistics methods and indicate statistical software which you used.

Response: Indeed, Fisher's LSD post hoc is restricted to 3 groups’ comparison. The LSD method does not control family-wise α error level. Therefore, it is inappropriate for multiple comparison procedure where the control of family-wise α error level is necessary (PMID: 25984481), Reference: Kim HY. Statistical notes for clinical researchers: post-hoc multiple comparisons. Restor Dent Endod. 2015; 40(2):172-6.) , and the LSD method can not be applied in the case of 4 groups or more. To examine significant differences among six experimental groups, Post-hoc multiple comparison procedures was conducted by the Bonferroni method and correction of α error was 0.001/15=0.003. We had added to describe in (Methods section, lines 26-29, Page 9): ‘ANOVA followed by Bonferroni post hoc multiple comparison was used to examine significant differences among the experimental groups. A adjust p-value<0.003 was considered to be significant [24].’ Statistic results had been added in Fig. 5, Fig. 6, Fig. 8 and Fig. 9 Legends and in results (Results section, lines 3-14, Page 11; lines 19-25, Page 11; lines 2-13, Page 12; lines 16-20, Page 12). We had added reference in (Reference section, lines 24-25, Page 22):

In addition, we had added to describe that the data are analyzed with SPSS 13.0 software (SPSS, Chicago, IL) in (Methods section, lines 28-29, Page 9): ‘All data was expressed as means ± standard deviation and analyzed with SPSS 13.0 software (SPSS, Chicago, IL).’

2. Although the levels of phosphorylated Smad1/5/8 and ALP activity were significantly increased in pcDNA3.1/BMPR-IA (MT -349C>T) vector-transfected C3H10T1/2 cells than the WT vector-transfected cells, the results would be overreaching to suggest "Smad signaling pathway plays a major role in the pathological process of OPLL induced by SNPs in BMPR-IA gene" in their conclusion.

Response: Yes, indeed. Although our data demonstrate that the levels of phosphorylated Smad1/5/8 and ALP activity were significantly increased in pcDNA3.1/BMPR-IA (MT -349C>T) vector-transfected C3H10T1/2 cells than the WT vector-transfected cells, this study have weakness in Vitro experiment and the results would be overreaching to suggest "Smad signaling pathway plays a major role in the pathological process of OPLL induced by SNPs in BMPR-IA gene" in conclusion. Therefore, we had rewrittten the conclusions in (Abstract section, lines 2-5, Page 3): ‘Our results suggest that Smad signaling pathway may play important roles in the pathological process of OPLL induced by SNPs in BMPR-IA gene. These results will help to clarify the molecular mechanisms underlying the SNP and gene susceptibility to OPLL’., and in (conclusions section, lines 17-24, Page 17): ‘The present results demonstrate that the expression levels of BMPR-IA gene, levels of phosphorylated Smad1/5/8 and ALP activity were significantly increased in pcDNA3.1/BMPR-IA (MT -349C>T) vector-transfected C3H10T1/2 cells than the WT vector-transfected cells. Our data suggest that Smad signaling pathway may play important roles in the pathological process of OPLL induced by SNPs in BMPR-IA gene. The current study may help to clarify the molecular mechanisms underlying the susceptibility of the gene to OPLL, thereby providing a novel potential target for the diagnosis and therapy for OPLL.’

3. In their In Vitro experiments, they used only normal media condition for the transfected cells. If they use some stimulation factors which would affect BMPR-IA during the cell culture, the transfected BMPR-IA function would be more understandable.

Response: Yes, indeed. In our in Vitro experiments, we use osteogenic induction medium containing 200ng/mL rhBMP2 as stimulation factor during the transfected C3H10T1/2 cell culture. However, we don't have a description in the manuscript. Therefore, we had added to describe in (Methods section, line 25-, Page 7, line 5, Page 8): ‘For stable transfection, cells were seeded at 4.5×105 cells/well in 6-well plates pre-coated with poly-l-lysine (Sigma, St Louis, MO, USA) and maintained in BME containing 10% FBS. After cells were grown to confluence, the gene transfections were performed using lipofectamine (Invitrogen) according to the manufacturer’s instructions. 4-6 hrs post-transfection, the transfected cells were cultured in osteogenic differentiation conditions with 200ng/mL recombinant human BMP2 (rhBMP2, BioSia, Co., Ltd, Shanghai, China) for 72 hrs. The following day, the cells were trypsinized (Gibco) and plated into a large cell culture dish. Stably transfected C3H10T1/2 cells were selected with 800 µg/mL G418 media for 2 weeks and subsequently maintained at 200 µg/mL G418 media (Gibco), following which, G418-resistant clones were selected and expanded.’
1. Please indicate sample size of this experiment in the Figure or Manuscript body.

2. In the (B), please add the explanation of "**" in the Lane 4 in the Figure Legends.

Response: 1. We had added sample size (n=5) of this experiment in the Figure (Fig. 5 section, lines 13): ‘n=5.’ and Manuscript body in (Results section, line 3 and line 19, Page 11): ‘n=5.’

2. In the (B), we had added the explanation of "**" in the Lane 4 in the Figure 5 Legends (Fig. 5 section, lines 13-29): ‘Fig. 5 (B) Statistical analysis of BMPR-IA protein expression levels in transfected C3H10T1/2 cells using the linear density ratio of BMPR-IA/GAPDH. The protein expression levels of BMPR-IA gene were significantly increased in C3H10T1/2/pcDNA3.1/BMPRIA (MT -349C>T, MT 4A>C, MT -349C>T and 4A>C) cells compared to the C3H10T1/2/pcDNA3.1/BMPRIA (WT) cells, C3H10T1/2/pcDNA3.1 cells, and C3H10T1/2 cells. n=5, *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA WT cells compared with C3H10T1/2 cells, *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA WT cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT 4A>C cells compared with C3H10T1/2 cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T and 4A>C cells compared with C3H10T1/2 cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T and 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA WT cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T and 4A>C cells compared with C3H10T1/2/pcDNA3.1/BMPRIA WT cells.’

Fig. 6

1. Please indicate sample size of this experiment in the Figure or Manuscript body.

2. In the (B), please add the explanation of "**" in the Lane 3 and 5 in the Figure Legends.

Response: 1. We had added sample size (n=5) of this experiment in the Figure (Fig. 6 section, line 15 and line 30): ‘n=5.’ and Manuscript body in (Results section, line 2, line 16, line 23 and line 24, Page 12): ‘n=5.’

2. In the (B), we had added the explanation of "**" in the Lane 3 and 5 in the Figure 6 Legends in (Fig. 6 section, lines 15-28): ‘Fig. 6 (B) Statistical analysis of phosphorylated Smad1/5/8 proteins levels in C3H10T1/2 cells, C3H10T1/2/pcDNA3.1 cells, C3H10T1/2/pcDNA3.1/BMPR-IA (WT) cells, and C3H10T1/2/pcDNA3.1/BMPR-IA (MT -349C>T, MT 4A>C, MT -349C>T and 4A>C) cells using the linear density ratio of
phosphorylated Smad1/5/8 proteins levels were significantly increased in C3H10T1/2/pcDNA3.1/BMPR-IA (MT -349C>T, MT -349C>T and 4A>C) cells compared to C3H10T1/2/pcDNA3.1/BMPR-IA (WT) cells, C3H10T1/2/pcDNA3.1 cells, and C3H10T1/2 cells. n=5, *p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA WT cells compared with C3H10T1/2 cells, *p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA WT cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT -349C>T cells compared with C3H10T1/2 cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1 cells; *p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT -349C>T cells compared with C3H10T1/2 cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1 cells; *p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT 4A>C cells compared with C3H10T1/2 cells, *p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1 cells; *p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT -349C>T and 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1/BMPR-IA WT cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA MT -349C>T and 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells. The protein levels of phosphorylated Smad1/5/8 were not increased significantly in C3H10T1/2/pcDNA3.1/BMPR-IA (MT 4A>C) cells compared to the C3H10T1/2/pcDNA3.1/BMPR-IA (WT) cells (n=5).'

In addition, we had also added sample size (n=5) of this experiment in the Figure 7 Legends and Manuscript body in (Results section, line 24, Page 12): ‘n=5.’ (Fig. 7 section, line 7): ‘Fig. 7 Western blot analysis of Smad4 protein levels in the transfected C3H10T1/2 cells. Lane 1 C3H10T1/2 cells; Lane 2 C3H10T1/2/pcDNA3.1 cells; Lane 3 C3H10T1/2/pcDNA3.1/BMPR-IA (WT) cells; Lane 4 C3H10T1/2/pcDNA3.1/BMPR-IA (MT -349C>T) cells; Lane 5 C3H10T1/2/pcDNA3.1/BMPR-IA (MT 4A>C) cells; Lane 6 C3H10T1/2/pcDNA3.1/BMPR-IA (MT -349C>T and 4A>C) cells. There was no significant differences in Smad4 protein levels among the experimental groups (n=5).’

Fig.8

1. Please indicate sample size of this experiment in the Figure or Manuscript body.

2. Please add the explanation of "**" in the Lane 3, and 5 in the Figure Legends.

Response: 1. We had added sample size (n=5) of this experiment in the Figure (Fig. 8 section, line 4 and line 22): ‘n=5.’ and Manuscript body in (Results section, line 2, line 6 and line 9, Page 13): ‘n=5.’

2. We had added the explanation of "**" in the Lane 3 and 5 in the Figure 8 Legends (Fig. 8 section, lines 4-19): ‘Fig. 8 The ALP activity in transfected C3H10T1/2 cells. The ALP activity levels were significantly increased in C3H10T1/2/pcDNA3.1/BMPR-IA (MT -349C>T 30.56±0.44 U/gprot, MT -349C>T and 4A>C 26.91±0.41 U/gprot) cells compared to C3H10T1/2/pcDNA3.1/BMPR-IA (WT 23.67±0.42 U/gprot) cells. n=5, *p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA WT cells compared with C3H10T1/2 cells, *p<0.001 C3H10T1/2/pcDNA3.1/BMPR-IA WT cells compared with C3H10T1/2/pcDNA3.1 cells;
**p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2 cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1 cells; *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT 4A>C cells compared with C3H10T1/2 cells, *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T and 4A>C cells compared with C3H10T1/2 cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T and 4A>C cells compared with C3H10T1/2/pcDNA3.1 cells; **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2/pcDNA3.1/BMPRIA WT cells, **p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T and 4A>C cells compared with C3H10T1/2/pcDNA3.1/BMPRIA WT cells. No differences in ALP activity in C3H10T1/2/pcDNA3.1/BMPRIA-IA (MT 4A>C 23.84±0.42 U/gprot) cells were observed compared to the C3H10T1/2/pcDNA3.1/BMPRIA-IA (WT 23.67±0.42 U/gprot) cells (n=5).’

Fig.9

1. Please indicate sample size of this experiment in the Figure or Manuscript body.

2. Please add the explanation of "**" in the Lane 3, 4, 5 and 6 in the Figure Legends.

Response: 1.We had added sample size (n=5) of this experiment in the Figure (Fig. 9 section, line 4 and line 19): ‘n=5.’ and Manuscript body in (Results section, line 13 and line 17, Page 13): ‘n=5.’

2. We had added the explanation of "**" in the Lane 3, 4, 5 and 6 in the Figure 9 Legends (Fig. 9 section, lines 4-15): ‘Fig. 9 The OC activity in transfected C3H10T1/2 cells. The OC activity levels were significantly increased in C3H10T1/2/pcDNA3.1/BMPRIA-IA (WT 1.01±0.10 ng/mL, MT -349C>T 1.04±0.12 ng/mL, MT 4A>C 1.02±0.09 ng/mL, MT -349C>T and 4A>C 1.03±0.13 ng/mL) cells compared to the control groups. n=5, *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA WT cells compared with C3H10T1/2 cells, *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA WT cells compared with C3H10T1/2/pcDNA3.1 cells; *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T cells compared with C3H10T1/2 cells, *p<0.001 C3H10T1/2/pcDNA3.1/BMPRIA MT -349C>T and 4A>C cells compared with C3H10T1/2/pcDNA3.1/BMPRIA-IA (MT 4A>C 23.84±0.42 U/gprot) cells were observed compared to the C3H10T1/2/pcDNA3.1/BMPRIA-IA (WT 23.67±0.42 U/gprot) cells (n=5).’
The objective of the authors was to describe the molecular mechanism of OCLL—a deliberating disease in which there is an ossification of the spine. Previous study from the lab recognized several SNP associated with OCLL, BMPR-1A is one of them. Here, the authors test whether the different SNPs in the BMPR-IA result in changes in Smad signaling in in vitro setting.

The manuscript is suitable for BMC and the findings are interesting. However, the conclusion that the authors arrive to is not supported enough by the experiments described. In addition, the manuscript is poorly written and difficult to read. I would recommend using a help from scientific writer or an English native speaker to rewrite the manuscript.

1-The manuscript should be written in more clear way.

Response: Indeed the manuscript is poorly written and difficult to read. Therefore, my manuscript has been strictly revised and rewritten according to reviewers' recommends and the grammar has been corrected by a professional editor of English language.

2-Focuses a lot on talking about receptor affinities but the studies do not address this aspect of BMP signaling.

Response: Indeed, we talk about BMPs of receptor affinities but the studies do not describe this aspect of BMP signaling. In our in vitro experiments design, four to six hours post-transfection, the transfected cells were treated with osteogenic differentiation media containing 200ng/mL rhBMP2 for seventy-two hours. The rhBMP2 act as stimulation factor to stimulate BMP signaling and affect the transfected BMPR-IA function. Therefore, we had added to describe in (Methods section, line 29-, Page 7, line 5, Page 8): ‘4-6 hrs post-transfection, the transfected cells were cultured in osteogenic differentiation conditions with 200ng/mL recombinant human BMP2 (rhBMP2, BioSia, Co., Ltd, Shanghai, China) for 72 hrs. The following day, the cells were trypsinized (Gibco) and plated into a large cell culture dish. Stably transfected C3H10T1/2 cells were selected with 800 µg/mL G418 media for 2 weeks and subsequently maintained at 200 µg/mL G418 media (Gibco), following which, G418-resistant clones were selected and expanded.’

3-More description about the in vitro cell cultures should be provide: were the cultures induced to differentiate?

Response: In our in vitro cell cultures, the osteoblastic differentiation on C3H10T1/2 cells were induced by osteogenic medium containing 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.2 mM ascorbic acid for 7–21 days. We had added to describe more details about the in vitro cell cultures in (Methods section, lines 13-20, Page 7): ‘The cells were cultured in basal medium eagle (BME, Gibco BRL, Grand Island, NY, USA) medium supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA). For osteogenic differentiation, cells were seeded at a density of 4.5×105 cells/well in 6-well plates and maintained in BME containing 10% FBS. Cells at 80% confluence were induced by osteogenic medium containing 100 nM dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma) and 0.2 mM ascorbic acid (Sigma) for 7–21 days. The culture medium was changed every 3 days and cells were grown at 37 °C in a humidified atmosphere containing 5% CO2.’
4-Please provide more details about the in vitro experimental design-when you transfect BMPR-IA into cell-line-what happens? Where the cells treated with differentiation media? How long were they cultured?

Response: In the in vitro experimental design, for stable transfection, cells were seeded at 4.5×105 cells/well in 6-well plates pre-coated with poly-l-lysine and maintained in BME containing 10% FBS. After cells were grown to confluence, the gene transfections were performed using lipofectamine according to the manufacturer’s instructions. Four to six hours post-transfection, the transfected cells were treated with osteogenic differentiation media containing 200ng/mL rhBMP2 for seventy-two hours. We had added to describe more details about the in vitro experimental design in (Methods section, lines 25-, Page 7, lines 5, Page 8): ‘For stable transfection, cells were seeded at 4.5×105 cells/well in 6-well plates pre-coated with poly-l-lysine (Sigma, St Louis, MO, USA) and maintained in BME containing 10% FBS. After cells were grown to confluence, the gene transfections were performed using lipofectamine (Invitrogen) according to the manufacturer’s instructions. 4-6 hrs post-transfection, the transfected cells were cultured in osteogenic differentiation conditions with 200ng/mL recombinant human BMP2 (rhBMP2, BioSia, Co., Ltd, Shanghai, China) for 72 hrs. The following day, the cells were trypsinized (Gibco) and plated into a large cell culture dish. Stably transfected C3H10T1/2 cells were selected with 800 µg/mL G418 media for 2 weeks and subsequently maintained at 200 µg/mL G418 media (Gibco), following which, G418-resistant clones were selected and expanded.’

Major concern

5-The authors concluded that changes in Smad signaling play major role in the pathophysiology of OPLL due to SNPs in BMPR-IA. However, there are not enough evidence to draw this conclusion. More studies should be done to show that those changes causing the pathological outcome.

Response: Yes, indeed. Although our data demonstrate that the levels of phosphorylated Smad1/5/8 and ALP activity were significantly increased in pcDNA3.1/BMPR-IA (MT -349C>T) vector-transfected C3H10T1/2 cells than the WT vector-transfected cells, there are not enough evidence to draw "Smad signaling pathway plays a major role in the pathological process of OPLL induced by SNPs in BMPR-IA gene" in conclusion. More studies should be done to show that those changes causing the pathological outcome. Therefore, we had rewritten the conclusions in (Abstract section, lines 2-5, Page 3): ‘Our results suggest that Smad signaling pathway may play important roles in the pathological process of OPLL induced by SNPs in BMPR-IA gene. These results will help to clarify the molecular mechanisms underlying the SNP and gene susceptibility to OPLL.’, and in (conclusions section, lines 17-24, Page 17): ‘The present results demonstrate that the expression levels of BMPR-IA gene, levels of phosphorylated Smad1/5/8 and ALP activity were significantly increased in pcDNA3.1/BMPR-IA (MT -349C>T) vector-transfected C3H10T1/2 cells than the WT vector-transfected cells. Our data suggest that Smad signaling pathway may play important roles in the pathological process of OPLL induced by SNPs in BMPR-IA gene. The current study may help to clarify the
molecular mechanisms underlying the susceptibility of the gene to OPLL, thereby providing a novel potential target for the diagnosis and therapy for OPLL.