Author's response to reviews

Title: BMP and TGFbeta Pathways in Human Central Chondrosarcoma: Enhanced Endoglin and Smad 1 Signaling in High Grade Tumors

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Author's response to reviews: see over
Reviewer 1: John Healey

General comments to the reviewer:

It is correct that the possible link between Smad1/5/8 activity, endoglin expression and angiogenesis is only a hypothesis and not proven by our study. This should not appear as the main conclusion of our study. This has been clarified in the revised version of the article. The mention to angiogenesis has been removed from the abstract and it is made clear in the discussion that this is only a hypothesis which needs further experiments to be proven (p.10).

Based on statistical analysis of current sample numbers, it does indeed not appear that endoglin or Smad activity could already be suggested as helpful diagnostic or prognostic tools. The analysis of larger patient cohorts would be necessary to establish this. We think our analysis rather gives insights into mechanisms that could be important for the progression of chondrosarcoma and highlights that the endoglin / Smad1/5/8 signaling axis is worth being studied further in chondrosarcoma, especially in regard of possible pharmacological targeting. This is also emphasized in our conclusion (p.11). To better clarify the limitation of restricted sample numbers in this study, the following was added to the discussion (p.11): “Since central chondrosarcoma is a rare tumor type and the isolation of good quality RNA is difficult due to low cellularity and extracellular matrix [31], one limitation of this study is the restricted number of samples which allowed reaching only levels of significance close to the threshold. The analysis of larger patient groups would be necessary to establish the robustness of the correlations found in this study and would be especially interesting to assess whether high endoglin expression significantly correlates to a high tumor vascularization and to a low metastasis-free survival.”

The number of samples used in our study made it indeed particularly difficult to analyze links between the BMP and TGF beta pathways and metastasis or lethality. Higher amount of high-grade tumors would be necessary to assess whether the correlations which we found to be close to significance could be confirmed. This is the reason why these aspects of progression linked to invasion, metastasis formation and lethality are not emphasized in the discussion of this study. The discussion is rather focusing on possible regulation mechanisms of Smad activity and the role this could play in the control of chondrogenic differentiation stages. The second paragraph of the discussion has been simplified for more clarity.
We agree that the cell line data can’t reflect on the endoglin axis directly, but we found it important to establish intact signaling pathways for Smad 1/5/8 and Smad2.

We apologize for incomplete description of statistical analysis and extended our section on statistical analysis (p.16). For the comparison of gene expression levels with the Mann-Whitney test, results without Bonferroni correction were used. Since the BMP family of ligands we study here is known for its capability to form active heterodimeric proteins and this cannot be judged by mRNA analysis, we considered a more system biology approach for these data as less helpful and went on to study Smad signaling.

We agree that a broader systems biology approach on the BMP and TGFbeta pathways in chondrosarcoma would be highly interesting and could reveal whether there are some links to angiogenic signaling pathways. Such a study could however not confine only on the ligands and receptor molecules like in this study.

Specific questions about the manuscript:

1. Introduction “a pivotal regulator providing a link between the undifferentiated phenotype of tumor cells in high-grade chondrosarcoma and the angiogenic status of these tumors.” This was not proven by this investigation, so it should not be emphasized here. Indeed, on page 6 you state “Only expression in tumor cells and not in the vasculature was scored in this study”

   We agree and removed comments on the angiogenic status of the tumors from the abstract.

2. P 4. “In central chondrosarcoma, active TGFbeta signaling has been shown according to nuclear phosphorylated Smad2 detection, and the expression of PAI1, a target gene of TGF#/

   The sentence has been changed to: “In central chondrosarcoma, TGFβ signaling is active according to detection of nuclear phosphorylated Smad2. A role of this pathway
in tumor progression was suggested as PAI1, a target gene of TGFβ/Smad2/3, showed higher levels in high grade tumors [10].”


We added that: “Endoglin was detected in the cytoplasm and on the membrane of tumor and vascular cells.”

4. P12. Was the RNA prepared at each center and then combined for analysis, or was the tissue processed centrally in the same lab?

Tissue samples were processed centrally in one lab. This was added to the page 12 methods section.

Reviewer 2: Nita Ahuja

1. The expression data mostly suggests that BMP levels and TGFB1-3 levels are decreased compared to normal. It is unclear why only certain BMPs were chosen?

Due to the low amount of mRNA available especially from low-grade chondrosarcoma samples, only a limited number of RT-PCR tests could be performed and a selection of genes from the BMP and TGFbeta pathways had to be performed. For the BMPs, only those known to play a prominent role in bone and cartilage formation were chosen (background, p.4).

2. However activity of Smad1/5/8 as detected by phosphorylated Smad 1/5/8 was higher in higher grade chondrosarcomas which was statistically significant. Similarly pSMAD2 was also seen most often in higher grade tumors, but not statistically significant. high pSMAD2 levels was also associated with shorter metastasis free survival on univariate analysis.
Similarly high endoglin expression (a coreceptor) also was associated with shorter metastasis free survival and increased Smad 1/5/8 signaling. However, figure 2F could show % rather than number of samples to better show this.

Figure 2F has been changed and numbers of samples are shown in % now.

3. However inhibition of activity of TGFbeta and Smad using in vitro inhibitors doesn’t affect proliferation. Does it affect apoptosis?

We have only analyzed cell viability / proliferation and did not see any changes after pathway inhibition. Therefore, we would not expect any apoptosis, but we did not analyze this. Our aim in this study was mainly to confirm the activity of the BMP and TGF beta signaling pathways in chondrosarcoma cell lines.

4. Although the authors attempt to explain increased activity of Smad1/5/8 due to relative increased expression of ALK2 or TFGbeta3 in grade 3 sarcomas the increased expression in grade 3 chondrosarcomas is relative since overall levels of ALK receptors and TGFbeta are down compared to normal. Clarification of this would be helpful.

The expression levels of ALK2 and TGFbeta3 in grade III chondrosarcomas are indeed similar to those in normal cartilage, while they are lower in grade I. We did however not analyze Smad activity in cartilage, so that no comparison can be made at this level between neoplastic and normal chondrocytes. Expression levels of many of the genes analyzed differ between cartilage and chondrosarcoma. Both pathways thus seem to be regulated very differently between chondrocytes and chondrosarcoma cells, and it is difficult to draw conclusions from expression levels of single genes, as the context in the pathways appear to be very different.

We have made clear in the discussion (p.8) that the hypotheses we make on regulation of Smad1/5/8 activity are based on comparisons between low and high-grade. Furthermore we have added in the discussion that: “Our gene expression profiles suggest that the BMP and TGF beta signaling pathways are regulated very differently between normal cartilage and chondrosarcoma.”