Author's response to reviews

Title: Anti-tumor effect of bisphosphonate (YM529) on non-small cell lung cancer

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Author's response to reviews: see over
Dear Editor:

Thank you so much for giving us opportunity to submit the revised version of our manuscript as an exceptional case. We are willing to submit our revised manuscript that has the additional results of western blotting requested by reviewer#1. We hope that our revised manuscript will be accepted in BMC Cancer as an original report.

Reviewer's report:

General
In their additional data of the revised Figure 4, YM529 apparently induce the expression of Ras (not only unprenylated Ras but also prenylated Ras). However, authors concluded YM529 inhibited Ras-ERK1/2 pathway. I cannot understand why the author concluded Ras-ERK1/2 pathway was inhibited by YM529 from their Western blotting result. They used Pan-Ras antibody (not unprenylated Ras antibody) in the Western blotting analysis. From their results, I understand the unprenylated Ras was increased by YM529. However, the prenylated Ras (functional form of Ras) also apparently increased in the expression. Their results are consistent with the previous report (Nogawa et al., Oncol Res 2005). Inhibition of geranylgeranylated proteins rather than farnesylated proteins may be important for the growth inhibitory effect of YM529 against NSCLC cells.

I know that recent reports have revealed that bisphosphonates prevent prenylation of the mevalonate pathway, which is essential to activate small GTP-binding proteins (G-proteins) such as Ras, as authors described in the Discussion section. Because nobody has demonstrated the inhibition of farnesylation and/or geranylgeranylation of the small G-protein in NSCLC cells, authors should demonstrate the prenylation inhibitory effect of YM529 in NSCLC cells. From their Western blotting analysis, I cannot find Ras-ERK1/2 pathway was inhibited by YM529.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached). Molecular target of YM529 remains unknown. I recommend authors should demonstrate the inhibitory of geranylgeranylation and/or farnesylation in NSCLC cells. It is very simple additional experiment.

Response:
We agree that the western blotting for Ras which we present in the revised version is
not appropriate because the expression of prenylated Ras (functional form of Ras) seems increase (It may be our problem of scan condition.). Actually, unprenylated Ras was increased by YM529 decreasing the prenylated Ras (For this revised version, we confirmed “Ras issue” by repeating experiments of western blotting). We also performed western blotting for Rap1A that is substrate of geranylgeranyl transferase to examine whether the geranylgeranylation is associated with inhibition of p-Erk1/2 or not. Our western blotting for Rap1A shows that YM529 induces the unprenylated Rap1A with dose dependent manner. Thus, we conclude that YM529 induce apoptosis by inhibition of both farnesylation and geranylgeranylation resulting in inhibition of p-Erk1/2.

In manuscript, we add or change following parts:

**Page8 line15.** “, anti-Rap1A (Santa Cruz Biotechnology inc., Santa Cruz, CA),”

**Page8 line18.** “then with goat anti-rabbit, goat anti-mouse and rabbit anti-goat IgG-HRP coupled to horseradish peroxidase conjugated secondary antibodies (Santa Cruz biotechnology inc., Santa Cruz, CA),”

**Page11 line2.** “we examined the protein expression status of members of the small GTP-binding protein related cascade that YM529 was assumed to inhibit in order to cause apoptosis. Western blotting analysis showed that anti-Ras or anti-Rap1A antibody recongnized the unprenylation of Ras or Rap1A in a dose-dependent manner of YM529 (Fig.4). These results indicated that YM529 inhibited Ras-ERK1/2 pathway via inhibition of both farnesylation and geranylgeranylation of GTP-binding proteins such as Ras and Rap1A. “

**Page12 line16.** “Since FPP and GGPP activation cause prenylation of small GTP-binding proteins including Ras resulting in anti-apoptosis, inhibition of their activation by YM529 induces cellular apoptosis.(25-29). Indeed, YM529 induced unprenylation of Ras and Rap1A resulting in down-regulation of ERK1/2 phosphorylation in NCI-H1819 cells despite absence of any effect on ERK1/2 expression”

**Page13 line8.** “small GTP-binding proteins associated signal transduction pathway. ”

*Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)  There is not Ras in the legend of Figure 4.*
We add Ras and Rap1A in the legend of Figure 4.

Best wishes,

Ryuichiro Koshimune, M.D.