Author's response to reviews

Title: Efficacy of Wnt-1 monoclonal antibody in sarcoma cells

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

Enclosed please find our revised manuscript entitled “Efficacy of Wnt-1 monoclonal antibody in sarcoma cells”. The manuscript has been modified considerably according to the helpful comments. We hope that this revised manuscript will be acceptable for publication in BMC Cancer.

Thank you very much for your consideration and I look forward to hearing from you in the near future.

Sincerely,

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Our responses to the comments:

For Reviewer #1:

1. Abstract: the authors described only methods used to evaluate apoptosis. They must describe also methods used in the experiments.

(Our response) We agree. We have added methods used in other experiments in the Abstract.

2. Materials and methods: please specify if experiments were performed only one time or more times to control the possibility of replication.

(Our response) Wnt-1 antibody and siRNA treatment was performed more than three times in our experiments. We have specified this in Materials and Methods.

3. There are some typographical errors (such as at pag. 6 line 5 substitute “(custom-made….).” with “(custom-made…),” or at line 7 “(Santa …).” with “(Santa …),”).

(Our response) We agree. We have corrected these typographical errors.


(Our response) We have inserted the reference at page 8.

For Reviewer #2

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

1. The authors discuss sarcoma as one disease identity (when in fact A204 is a rhabdomyosarcoma cell line) and then report conclusions as if they would apply to all sarcomas. Sarcoma is a general classification for tumors originating from mesenchymal tissue. Each type of sarcoma has different pathologic mechanism and responds to different treatment modalities. Results from a single cell line cannot be generalized to the whole family of sarcomas.

(Our response) We agree with the reviewer that results from one cell line cannot be generalized to the whole family of sarcomas. We have treated another sarcoma cell line SJSA-1 (bone sarcoma) expressing Wnt-1, with the antibody and found similar results to A-204 cell line. The results for SJSA-1 cells have been added in the manuscript. We have also corrected the statement and conclusions in the Abstract and Discussion to: ”the Wnt-1 antibody may have therapeutic roles in the treatment of a subset of sarcoma cells in which Wnt-1/beta-catenin signaling is active”.
2. Patient samples were described as lung metastasis of sarcoma. What was the primary tumor pathology for these lesions? The whole tumor tissue was homogenized and used in cell culture experiments. The results given in Figure 1 and Figure 4 are therefore difficult to interpret since there is no way of knowing if the effect is on tumor or stroma. Furthermore, western blots shown in Figure 1 do not prove that these cells have an active (autocrine or paracrine) Wnt signaling. The presence or absence of beta-catenin and cyclin-D1 could result from many cell signaling processes and are not specific to Wnt-1. In order to be convincing, Wnt-1 treatment with and without antibody are necessary to support the cause/effect relationship with these primary tumor cells.

(Our response) We have added the primary tumor pathology information in the legend of Figure 1. In fact, the primary cultures treated with control and Wnt-1 antibodies presented in Figure 4 are the cases 3 and 6 presented in Figure 1 (We have omitted the figure of primary culture 3 because of the subtype is unknown.) We have recently reported that the Wnt-1 antibody induces apoptosis in human colorectal cancer cells. (Please refer to He B, et al. Oncogene 2005, doi: 10.1038/sj.onc.1208511.) We agree with the reviewer that Cyclin D1 could result from many cell signaling processes. We have omitted Cyclin D1 from the manuscript. However, upregulated cytosolic beta-catenin levels are one of the most important indicators for the activation of canonical Wnt pathway. Therefore, we feel that a relation exists between Wnt-1 and cytosolic beta-catenin.

3. The Wnt-1 antibody is problematic due to a lack of binding and specificity validation. Even though the monoclonal Wnt-1 antibody has been published earlier, neither this manuscript nor the original one addresses the issue of cross reactivity. This antibody was raised against a 12 aa long peptide. Wnt-3, Wnt-5a, Wnt-8b, and Wnt-16, all have similar domains with 8-9 identical aa. This clearly taints the conclusions of the entire paper. Specifically, the western blot in Figure 1 is not convincing. There are multiple bands and high background. In addition, peptide competition of antibody effects would have helped to support Wnt-1 specificity.

(Our response) This is a good point. To address the issue of cross reactivity, we have recently shown (please refer to He B, et al. Oncogene, 2005, doi: 10.1038/sj.onc.1208511) that the growth of non-small cell lung cancer cell line A549, which expresses Wnt-2 but not Wnt-1, was not affected by anti-Wnt-1 antibody. However, an anti-Wnt-2 antibody that we developed (You L, et al. Cancer Res 2004, 64:5385-9) killed the A549 cells. In addition, we found that the anti-Wnt-1 antibody precipitated Wnt-1 proteins in C57Wnt-1 cells (He B, et al, Neoplasia 2004; 6:7-14), but it did not precipitate Wnt-2 proteins in C57Wnt-2 cells or Wnt-3 proteins in C57Wnt-3 cells (unpublished data). Taken together, these results suggest that the anti-Wnt-1 antibody used does not likely cross react with other Wnt family proteins, such as Wnt-2 and Wnt-3. Peptide competition of the Wnt-1 antibody effects has been reported previously (He B, et al. Neoplasia 2004, 6:7-14), supporting the Wnt-1 specificity. Finally, we have repeated western blot analysis for Wnt-1 in Figure 1 and replaced with a clearer result. The multiple bands could be due to the nature of the Wnt-1 proteins (differential glycosylations).
4. Figure-3b shows results of an siRNA experiment. However, the figure lacks a control, Wnt-1 western blot, to show that siRNA works in this cell line. Earlier published work on other cell lines does not guarantee the outcome in this particular cell type. The effect on beta-catenin is very limited compared to Figure-3a.

(Our response) We agree. We have conducted the experiments suggested and found in western blot that Wnt-1 protein level was downregulated by Wnt-1 siRNA treatment in A-204 cells. The result has been included in new Figure 3B. Our interpretation for the limited effect on beta-catenin after siRNA treatment is that siRNA only knocks down mRNA, but not proteins. Therefore, secreted and cell surface Wnt-1 proteins may still function even after siRNA treatment, which may attenuate the siRNA’s knock-down effect. We hypothesize that higher dose siRNA and/or longer treatment may have a greater effect on beta-catenin level. Indeed, we found that this is the case. More apoptosis induction and beta-catenin downregulation after higher dose siRNA treatment were observed. We have included these new results in new Figure 3A and 3B.

5. Apoptosis is described based upon only one Annexin V binding study and the results lack information about PI uptake as a control for non-apoptotic cell death.

(Our response) We agree. We have included the results about Annexin V and PI double staining according to the reviewer’s helpful suggestion (please see Figure 3A).

6. The abstract is poorly written.

(Our response) We agree. We have modified the Abstract accordingly.

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Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

1. Origin of A-204 cell line, an embryonic rhabdomyosarcoma, should be given.

(Our response) We agree. We have included the origin of A-204 cell line in the manuscript (please see Materials and Methods).

2. To eliminate the possibility of simple general toxicity, the Wnt-1 monoclonal antibody should be tested on a cell line that does not express any Wnt-1.

(Our response) We agree with the reviewer. In fact, in our previous reports, we have shown that the anti-Wnt-1 antibody does not kill cells lacking the Wnt-1 protein (both cell lines and primary cultures) (please refer to He B, et al. Oncogene 2005, doi: 10.1038/sj.onc.1208511 and He B, et al. Neoplasia 2004, 6: 7-14), suggesting that the Wnt-
1 antibody does not likely induce simple general toxicity. We have added the two references in the Introduction of the manuscript.

3. The sequence of the siRNA and protein effects should be shown.

(Our response) We agree. We have included the sequence of the siRNA in the Materials and Methods. We have also conducted the experiments and found that Wnt-1 protein level was downregulated by Wnt-1 siRNA treatment. The result has been included in Figure 3B.

4. Whenever a cell fractionation is done (Figure-1 and Figure-3), it would be best to show the whole cell lysate as a control.

(Our response) We agree with the reviewer. However, upregulated cytosolic beta-catenin levels are critical indicator for the activation of canonical Wnt pathway (Ref. Lustig B, et al. Cancer Res Cin Oncol 2003, 129:199-221, Van Es JH, et al. Curr opin genet Dev 2003, 13:28-33, Nelson WJ, et al. Science 2004, 303;1483-1487). Therefore, it is more relevant to our studies here and we feel that it may not be necessary to include beta-catenin level in whole cell lysate.