Author's response to reviews

Title: Patient-derived heavy chain antibody targets cell surface HSP90 on breast tumors

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

We once again thank you for considering our manuscript "Patient-derived heavy chain antibody targets cell surface HSP90 on breast tumors" (Manuscript ID: 1335336353163851) for potential publication. We also thank the reviewers for their excellent and constructive comments and we have revised our manuscript to answer the questions raised by both the reviewers. Importantly, we have performed additional experiments that were requested by both reviewers, namely transwell migration series. We believe that this significantly improves the manuscript, provides additional support for our cell surface targeting antibody, and further completes this body of work. Below we have listed the comments provided by the reviewers as well as our responses and/or changes pertaining to each of the comments.

Reviewer 1 comments:

“The manuscript is well read and has some revelatory implications in as much as the power of the strategy to screen antibodies is concerned. However, the applicability of the antibody as an anti-tumor agent is not strongly evident and stronger assays are needed to validate its relevance alongside other HSP90 inhibitory antibodies or small molecule inhibitors. More powerful migration assays like transwell/boyden chamber assay alongside suitable in vivo metastasis model are needed to validate the potential of the antibody to inhibit migration. Moreover a positive control is missing.”

Response: We performed in vitro transwell migration assay and the results have been incorporated in the revised manuscript (Figure 5H). We included 17-DMAG (HSP90 inhibitor) as a positive control in the assay as well as a non-cell surface targeting commercial anti-HSP90
antibody as a negative control. The potential of HCAb2 in inhibiting *in vivo* metastasis will be examined in the future and we believe is beyond the scope of this primary manuscript.

“Although the antibody seems to accumulate inside the xenograft tumor mass, it is not clear whether it has any potential to restrict tumor growth by activating immune effector mechanism like ADCC and CDC. The mere accumulation of an antibody does not suggest restriction of tumor growth.”

**Response:** This manuscript is a proof of principle manuscript that establishes the efficiency of our novel antibody generation and screening strategy. The outcome of our screening strategy led to the identification of HCAb2 that targets cell surface HSP90 on breast tumors in comparison to normal tissues. We also showed that HCAb2 binds to MDA-MB-231 xenograft tumor cells in comparison to mouse normal tissues. We agree that this is a seminal point; however a complex approach is required to definitively evaluate tumor growth restriction properties of HCAb2. Our future experiments will be aimed at exploiting the preferential binding of HCAb2 to tumor tissues, which include performing both CDC and ADCC assays. We also plan to conjugate drugs/toxins to HCAb2 and determine therapeutic potential of HCAb2 in an *in vivo* setting. Clearly, this is an expansive plan and would require data well beyond this proposed manuscript.

“Toxicity profile of the antibody is needed to determine the optimum dose. Also needed to determine is the stability of the antibody.”

**Response:** As mentioned above, this is a proof of principle manuscript and subsequent in depth analysis on different parameters *in vitro* and *in vivo* will be performed in future experiments. In all these studies performed here, no toxicity was evident either by animal weight, activity or
tissue assessments. Optimization of both dose and potential activity is again an extensive plan and will be included in future comprehensive experiments.

“The study validates the potential of the technique developed by the author but since the other major objective of the study is to determine the tumor growth restriction property of the antibody, the study needs more elaboration regarding the therapeutic potential of the antibody.”

Response: Please see previous response.

Reviewer 2 comments:

“Authors claimed HCAb2 could affect migration of MDA-MB-231 cells by performing an in vitro scratch assay in presence of HCAb1 and HCAb2 antibodies in comparison to untreated controls (Figure 5). However, it is not easy to get such impression based on data provided by authors; it will be more helpful if they could perform other assays, such as Western blot for some typical bio-markers of cell migration or perform transwell assay to validate this. In addition, it would be a good control if HSP90 inhibitor was used.”

Response: We performed an in vitro transwell migration assay and the results have been incorporated in the revised manuscript (Figure 5H). We included 17-DMAG (HSP90 inhibitor) as a positive control and a non-cell surface targeting commercial anti-HSP90 antibody as a negative control in the assay.
“In Figure 4B, IP with HCAb1 followed by WB with HSP90(b) also showed a faint band at the same position as that strong band from IP with HCAb2 and WB with HSP90(b), in considering bottom panel of HAbs, the lane of IP with HCAb2 has much more abundant protein, I would suspect that faint band on the lane of IP with HCAb1 is the same one in the lane of IP with HCAb2, meaning HSP90(b) was in the precipitation for both HCAb1 and HCAb2, what would authors explain this?”

Response: Since the immunoprecipitation was performed with purified recombinant HSP90β protein, we believe that a minor non-specific pull down was seen with HCAb1, while HCAb2 pulled down a larger specific amount of HSP90β. Comment to this effect was provided in text; Lines 443-445.

The reviewer also suggested that there was more HCAb2 compared to HCAb1 in the immunoprecipitations. From Figure 4B, it appears that there is more HCAb2 than HCAb1 in the pull-downs. However, we have confirmed that this is due to the intensity of anti-mouse recognition of HCAb2 compared to HCAb1 and not due to the difference in total amounts. Below we show a figure (Figure 1) with a defined amount of each antibody by Coomassie staining and immunostaining with anti-mouse antibody for comparison. Clearly, the HCAb2 antibody runs in SDS-PAGE with some trailing which intensifies the immunostaining, despite being essentially the same total protein amount. In conclusion, we believe that the faint band seen with HCAb1 in Figure 4B is a minor non-specific interaction between HCAb1 and HSP90β and the levels of both HCAb1 and HCAb2 in the immunoprecipitation were equal.
“Line 78, “Although successful,”?”

Response: Edited Lines 77-80 to clarify.

“Line of 319, HCAb2 was incubated with permeabilized cells (Fig 1 I-O) should be (Fig 1 M-O).”

Response: Edited Line 335 and corrected to Fig 1M-O.

“It would be appropriate if HMEC cells were used as a control in xenograft model.”

Response: HMECs are normal primary human mammary epithelial cells that will not grow as xenografts in mice. In fact they are rapidly depleted when implanted as a cell suspension within several days and thus cannot be used as same time-frame controls. We have considered implantation within matrigel to support their survival but the matrix environment and angiogenic response is quite different and may not be an appropriate control for aggressive tumor lesions in
situ. We did take significant steps to determine HCAb2 specificity in the host by evaluating various mouse normal tissues. As shown in Figure 6F-J, HCAb2 did not localize to any mouse normal tissues but preferentially localized to the MDA-MB-231 xenograft. Also, flow cytometry analysis on MCF10A cells as well as immunofluorescence analysis on HMECs, demonstrated that HCAb2 specifically binds to the cell surface of MDA-MB-231 cells and similarly localizes to tumors in vivo. We believe this is comprehensive and appropriate for this study. Future in vivo studies will be aimed at determining selectivity between different breast tumor types.

“In Fig 7. J-L, one bright cell would be enough to support authors assumption”

Response: We have replaced Figure 7J-L with images that show patches of cells demonstrating HCAb2 localization despite similar levels of total HSP90 in xenograft tumor cells. Thus, HCAb2 targets a cell surface HSP90 antigen represented in a subset of the tumor cells.

We appreciate your consideration of this revised manuscript for publication and appreciate the improvements suggested by the reviewers.