Author's response to reviews

Title: Cystatin E/M suppresses legumain activity and invasion of human melanoma

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Version: 3 Date: 21 December 2009

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

Thank you for reviewing our revised manuscript MS 1849254872269013, entitled “Cystatin E/M suppresses legumain activity and invasion of human melanoma” by Jon J. Briggs, Mads Haugland Haugen, Harald Thidemann Johansen, Adam I. Riker, Magnus Abrahamson, Øystein Fodstad, Gunhild M. Mælandsmo and Rigmor Solberg.

We were very pleased to see that Reviewer 1 now considers the manuscript ready for publication and that the Associated Editor feels that no additional experiments are needed for the publication to be accepted. Although we have received new comments from Reviewer 2, we are pleased to see that the reviewer has changed the level of interest from “An article of limited interest” to “An article of importance in its field”. As requested, we have included the protease activity data for legumain, cathepsin B and L in a new figure (Figure 3), and previous figures 3 and 4 are now labeled figures 4 and 5, respectively.

Below we address the comments of Reviewer 2. Notably, Reviewer 2 has now raised new questions and comments to the data presented in the original manuscript, and we are pleased to observe that the reviewer now seem to better have understood the presented data. The reviewer is also repeating some comments originally addressed by Reviewer 1 and thus answered in our previous submission.

Reviewer 2:
Major Compulsory Revisions:
Unfortunately, the revised MS by Briggs et al. has not really improved. Although parts of the text were changed to make some of the statements clearer, there are no additional data presented which would support a causal relationship between cystatin E/M secretion and the metastatic potential of melanoma cells. The whole study remains descriptive.

Author’s comments: We agree that the screening of cystatins and cysteine proteases in the panel of melanoma cell lines are mainly descriptive. It has never been our intention to draw conclusions between the expression of cystatins/cystein proteases and the metastatic capacity of different melanoma cell lines. The metastatic cascade is far too complicated for such comparisons. What we have done is to use the Boyden chamber Matrigel invasion assay to evaluate the invasive capacity in cell lines over-expressing cystatin E/M, and have observed that enhanced expression of the protease inhibitor suppresses the invasive capacity (now Fig. 5).
While the authors clearly show that overexpression of cystatin E/M in two cystatin E/M non-expressing metastatic melanoma cells (A375 and MCC11) results in the inhibition of legumain activity (Fig. 3C/D) and a consequent decrease in the invasion capacity of the cells in the Matrigel gel assay (Fig. 4), the authors do not even attempt to demonstrate that the metastatic melanoma cell lines secreting massive cystatin E/M endogenously (MCC57 and MCC72) have a lower invasive capacity than the cell lines where no secreted cystatin E/M was detectable (MCC11, MCC35, MCC52, MCC69B). The authors argue that the Matrigel assay is too expensive to be performed for all cell lines. In addition they state that measuring the invasive capacity in the whole panel of cell lines will not necessarily reflect the protease/inhibitor in question, but rather the total levels of proteases and inhibitors in the different cell lines. I do not think it would not be too costly to just compare two high and low cystatin E/M-producing metastatic cell lines. Moreover, even though other protease/inhibitor pairs may contribute to the metastatic potential, the secretion of massive amounts of cystatin E/M (as it is the case for MCC57 and MCC72) should clearly diminish this potential because it blocks legumain, a crucial metastasis promoting protease.

Author’s comments: This issue was raised by Reviewer 1 and was answered in the previous revision of the manuscript.

Additional points:
1) The positive correlation between high cystatin E/M secretion and low residual legumain activity in the conditioned media assay does not hold true for all cell lines. For example, MA11 cells show high cystatin E/M levels but almost no legumain inhibitory activity (Fig. 1A/B). Conversely, the media of MCF10A, 92.1 and 624.38 cells exerts relatively good legumain inhibition, but no cystatin E/M secretion. The legumain activity data do not even correlate with cystatin C (which could replace cystatin E/M) because 92.1 cells do not secrete this protein while the other two cell lines do (MCF10A and 624.38). Moreover, the metastatic cell line MCC52 and MCC57 display similar legumain inhibitory activity, but only the latter cell line really expressed cystatin E/M.

Author’s comments: The statistical correlation analyses were performed on all cell lines as a group, showing a positive significant p-value. In this respect, comparing secretion from each cell line and its corresponding inhibitory capacity of exogenous legumain do not necessarily correlate.

2) The authors promised to show some human melanocytes as control lines (apart from HDF and HaCaT) but none are really presented in the revised MS (see author's comments to point#2).

Author’s comments: Originally, only HDF was included. As previously commented and shown in the revised fig. 2 (Skin; lower panels), we included the expressions of legumain, cathepsin B and L in cell lysates from both human melanocytes (Mel) and the human keratinocyte cell line HaCaT. Unfortunately, we were not able to find a supplier of conditioned media from melanocytes, but we were able to analyze the cystatins and inhibitory capacity towards exogenous legumain in conditioned media from keratinocytes (previously shown in revised Fig. 1; lower panels, Skin, HaCaT).

In the Discussion (page 22) we also wrote: “To confirm that CST6 functions as a tumor suppressor gene in melanoma, further studies are needed to determine the extent of cystatin
E/M expression and the glycosylation status in cultured melanocytes and thin primary tumors. Although the expression of cystatin E/M in melanocytes has not been demonstrated, and: Herein, we show that cystatin E/M is secreted by HaCaT keratinocytes, whereas no cystatin E/M was observed in lysates of either melanocytes or HaCaT cells."

3) I do not buy the argument of the authors that the difference of the ELISA (Table 1) and Western blot data (Fig. 1A) is due to the possibility that in the ELISA assay part of the cystatin E/M is bound to the legumain protease. Then you should be able to show that legumain and/or cathepsin B or L are secreted. At least for legumain this does not seem to be the case (as stated in the last sentence on page 18 (cathepsin B and L activities were not shown). Thus, it remains peculiar that cystatin E/M can be detected by ELISA in the MCC70, but not MCC13, 57 and 72 cell lines although all cells have a low expression level of legumain, cathepsin B and L (Fig. 2).

**Author’s comments:** This has been thoroughly discussed in the original manuscript and commented previously.

4) It is very interesting that most of the analyzed cell lines which secrete lots of cystatin E/M (with the exception of HCT-116) also exhibit low levels of processed, active legumain, cathepsin B and L. The authors interpret this correlation as an inhibition of the protease processing/activities by cystatin E/M. However as the inactive proforms of these proteases are also diminished (Fig. 2), an alternative explanation could be that cystatin E/M and cathepsin B/L expression levels are counterregulated by some unknown mechanism. In this case the lower metastatic potential of the cystatin E/M expressing cells might also be due to lower total expression levels of legumain, cathepsin B and L. This possibility should be envisaged and discussed.

**Author’s comments:** We thank the reviewer for this highly relevant suggestion and some comments regarding this possibility have been included in the Discussion section (page 24).

This is why the authors have to perform the Matrigel assay on the metastatic cell lines. If cystatin E/M expressing cells migrate faster, overexpression of legumain or cathepsin B/L and underexpression of cystatin E/M by shRNA will distinguish between the importance of the protease expression as compared to inhibitor levels.

**Author’s comments:** Again, cells that are expressing cystatin E/M migrate slower (not faster) than cells with no cystatin (see Fig. 5 and previous author’s comments to Reviewer 2). In addition, others have already shown that silencing of cystatin E/M markedly increased proliferation rate, in vitro motility and Matrigel invasiveness, as previously mentioned (Discussion, page 25).

5) There is another discrepancy between the cellular legumain activity (shown in the referees comments part) and the Western blot data of the processed, active protease form. While the amount of the active 36 kD form of legumain is comparable between the DU145, MCF10A, MCC13, MCC70, MCC57 and MCC72 cell lines, the legumain activities tremendously vary. Why? I do not understand this, especially because legumain activity/protein does not seem to be lost from the cell (secreted).
Author’s comments: As mentioned previously, no active legumain was detected in conditioned cell media. In the Western analyses, all amount of the particular protease is seen. In the activity analyses, only the free and active protease in measured. If the protease is bound to an endogenous inhibitor or adaptor protein (known or unknown) this could be reflected in the variations between expression and activity level within one cell type. Nevertheless, the linear regression analysis on the whole panel of cell lines showed a positive correlation between expression and activity of legumain (and cathepsin B, but not cathepsin L).

6) The data obtained from legumain, cathepsin B and L activity measurements using cleaved peptide substrates should be shown in the MS (not only mentioned on in the last paragraph on page 18).

Author’s comments: Enzyme activity data for all three proteases has now been included in a new figure 3 (A, legumain; B, cathepsin B; and C, cathepsin L).

We hope that the new revised manuscript is now acceptable for publication in BMC Cancer. The manuscript is not published or under consideration for publication elsewhere. The manuscript in its present form has been approved by all authors.

We are looking forward hearing from you.

Sincerely yours,
Rigmor Solberg