Author's response to reviews

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

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

Thank you for reviewing our 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 have taken the reviewers comments into consideration, have performed additional analyses and revised the manuscript accordingly. We feel that this has raised the quality of the manuscript. Below we address the reviewers’ specific comments and concerns.

**Reviewer 1:** Thomas Reinheckel (Albert-Ludwigs-Universität Freiburg, Freiburg, Germany)

We are pleased to read that the reviewer finds that “the topic of this article is timely and interesting”, that it “could nicely contribute to the literature on proteases/protease inhibitors in cancer” and that the level of interest is “an article of importance in its field”.

**Major concerns and authors comments:**

1) Figure 1 shows an association of cystatin M/E expression with legumain activity in cell culture supernatants of a panel of cell lines. However, some cell lines shown in A are missing in the activity measurements in B. Why are these cells omitted? Results need to be shown.

**Author’s comments:** In Fig. 1B, we showed the inhibitory ability of conditioned cell media towards legumain (partially purified from rat kidney) in all the cell lines showing expression of cystatin E/M by immunoblotting. In addition, some cell lines where cystatin C but not E/M were detected were also included (in total 11/18 melanoma and 4/6 non-melanoma cell lines). We think that the results clearly demonstrated proof of principle and illustrated the active nature of the cystatins secreted from melanoma cell lines. Nevertheless, we have now revised Fig. 1B and included results from analyses in the additional conditioned media.

2) The results of Figure 1 are hard to interpret in the context of the results shown in Figure 2. Cell lines MCC13, MCC57, and MCC72 show low legumain activity and high cystatin M/E (Fig. 1) but also very low legumain protein level in cell lysates. Hence, it is not clear if the low legumain activity in the cell culture media is due to inhibition by cystatin M/E or due to low expression and secretion of the protease. To solve this specific legumain activity
(legumain activity / legumain protein concentration) needs to be determined in the conditioned media.

**Author’s comments:** We realize that the results presented in figure 1 could be misinterpreted, and we have rephrased the explanation in the figure legend. In figure 1A we show cystatin E/M secretion to conditioned media by immunoblotting. In figure 1B the inhibitory activity towards legumain was evaluated by measuring the ability to inhibit legumain activity (a preparation of legumain isolated from rat kidney) after addition of conditioned media. The activity of the legumain preparation alone was used as control (Fig. 1B, Control, 100 %). The bars for each cell line actually show residual activity of legumain compared to legumain alone (Control). We see now that the text could be misleading when referring to Fig. 1B since the bars do not show inhibition but rather “residual activity” of legumain. The text on the y-axis in Fig. 1B has now been changed accordingly to make the presentation of these results clearer. Conditioned media from MCC13, MCC57, and MCC72 contained high levels of secreted cystatin E/M shown by strong cystatin E/M bands on immunoblots (Fig. 1A) and high inhibitory activity against rat legumain presented as low residual legumain activity (Fig. 1B).

Legumain activity has also been analyzed in conditioned media but no active legumain was detected. It is not expected that legumain could be active as an enzyme with the pH-conditions present in media. Thus, no active secreted legumain has been detected and this result has been included in the revised manuscript (page 18).

Further, we want to point out that the results in Fig. 1B and Fig. 2 cannot be compared, since the inhibitory effect of media is performed on equal amounts of purified legumain added to the assays (Fig. 1B), whereas the immunoblots show the endogenous expressed legumain in the cells (Fig. 2). In addition, in the original manuscript we mentioned that linear regression analysis of legumain immunoblots and activity in the cell lysates revealed a positive correlation between the putative active 36 kD form and activity of legumain (for detailed legumain activity results, see figure below; reviewer 2, comment 6).

3) To strengthen to point of the paper, the protease and cystatine levels should be further correlated with the invasiveness of the cell lines measured by Matrigel assays (as presented in Figure 4).

**Author’s comments:** We agree that it could be interesting to correlate the protease and cystatin levels with invasive capacity in additional cell lines, but have chosen not to prioritize this of the following reasons. The invasive capacity of tumor cells depends upon the total balance between different proteases and their specific inhibitors, of which the cysteine proteases (cathepsins/legumain) and the cystatins constitutes one of several protease families and their inhibitors, respectively. Measuring the invasive capacity in the whole panel of cell lines will then not necessarily reflect the protease/inhibitor in question, but rather the total levels of proteases and inhibitors in the different cell lines. Being afraid of getting results difficult to interpret and since the Matrigel assay is costly and time consuming we therefore decided to use cell lines not expressing basal levels of cystatin E/M and address whether the invasive capacity and protease activity (legumain) changed upon over-expression of the protease inhibitor (cystatin E/M).
4) Cathepsin L has been shown to interact with cystatin M/E at a site distinct from legumain. Furthermore extensive work from Frade and colleagues suggests cathepsin L involvement in melanoma. Hence it would be highly reasonable to include cathepsin L activity measurements & western blots in the analyses of Figures 1 and 2; but especially in the more functional investigations presented in Figures 3 and 4.

Author’s comments: We agree that cathepsin L is an interesting protein in relation to skin, melanoma and cystatin E/M. We have now performed immunoblots on cathepsin L on all the cell line lysates included in this study. In addition, cathepsin L activity has been measured in the cell lysates by using the peptide substrate Z-Phe-Arg-NHMec in the presence of the cathepsin B-specific inhibitor CA074 and the cathepsin L-specific inhibitor Z-Phe-Tyr(t-Bu)-diazomethylketone. The results have been included in the revised manuscript (page 18 and Fig. 2).

Unfortunately, it has not been possible to measure the cathepsin L activity in the CST6-transfected melanoma cells, since there is no more left of these samples. It does not seem rational to perform cell culturing and the analyses of Fig. 3 and 4 all over again (including cathepsin L activity) of the following reasons: The inhibitory constant (K\textsubscript{i}) of cystatin E/M versus legumain, cathepsin B and L is 0.0016 nM, 32 nM and 1.78 nM, respectively (MEROPS database; http://merops.sanger.ac.uk/). In the cystatin E/M over-expressing melanomas we do not know how much of the inhibitor the cells are producing, but it is in a range that has no effect on cellular cathepsin B, whereas cellular legumain is significantly but not fully inhibited (Fig. 3C). Given this, it seems reasonable that the concentration of cystatin E/M is not sufficiently high to result in effective cathepsin L inhibition, since the K\textsubscript{i} value for cystatin E/M inhibition of legumain is approximately 1000-fold lower than that for cystatin E/M inhibition of cathepsin L.

Reviewer 2: Christoph Borner (Albert-Ludwigs-Universität Freiburg, Freiburg, Germany)

We recognize that the essence of our manuscript could have been difficult to interpret, as the reviewer repeatedly refer to an increased invasion/migration of cystatin E/M over-expressing melanoma cells, whereas we both in the original title, text and figure 4 of the manuscript showed the opposite. We have now carefully revised the manuscript and hope that this is now clearer.

Major points:

1) Only secreted cystatin E/M and C levels are examined, but not how much of the inhibitors remain inside the cells.

Author’s comments: The intracellular levels of both cystatins have been investigated by immunoblotting of all the cell lines included. No immunobands were obtained indicating that the levels of cystatin E/M and C were below the level of detection. These results have now been included in the revised manuscript (page 16).

2) Control cell lines (non-cancerous skin cells) are missing.
Author’s comments: We have now performed analyses on human melanocytes and the human keratinocyte cell line HaCaT. Also, in the original non-melanoma cell panel, human dermal fibroblasts (HDF) were included. Immunoblotting and enzyme activities have been performed on melanocyte/keratinocyte cell lysates for legumain, cathepsin B and L. In addition, immunoblotting of cystatin E/M and C, as well as inhibitory capacity towards legumain, has been performed on conditioned media from keratinocytes. These results have been included in the revised manuscript (page 15, Fig. 1 and 2).

3) Not even half of the primary or metastatic cell lines exhibit increased cystatin E/M secretion. This is statistically insufficient. Who tells us that there is not a natural variation in the system? Also, increased cystatin E/M expression does not necessarily mean increased metastatic potential because it is also seen in 2 out of 5 primary melanoma cell lines which do not invade.

Author’s comments: It is somewhat hard to understand what the reviewer means by this comment. A natural variation does seem to occur among the melanomas and this might have functional consequences. Cystatin E/M has previously been considered as a tumor suppressor which means that high cystatin E/M levels results in low (not high) metastatic potential and vice versa. In fact, in the original manuscript we showed that increased cystatin E/M expression after CST6-transfection both inhibited cellular legumain activity and lowered (not increased) the cells potential to invade Matrigel compared to controls. Also, we do not generalize but suggest that when a cell expresses and secretes more of the inhibitor (cystatin E/M) the cellular enzyme activity (legumain) is reduced and then the cell is less invasive, which adds to previous observations in other cancer types. We hope that the revised manuscript clarify this.

4) While there is a good correlation between the immunoblotting and ELISA assays for cystatin C, this is not the case for cystatin E/M in the MCC13, 75 and 72 cell lines: Cystatin E/M is nicely detected by immunoblotting, but not by ELISA.

Author’s comments: This issue was thoroughly discussed in the original manuscript as cited from the Discussion section (page 22): “By immunoblotting, we detected cystatin E/M secretion from four melanoma cell lines (MCC13, 57, 70 and 72), and the conditioned media from these cells demonstrated the highest inhibitory activity against legumain. Surprisingly, we detected cystatin E/M by ELISA in media from only one of these melanoma cell lines. There are several potential explanations for this discrepancy, but the most likely being that there are fundamental differences between the applied methods. Immunoblotting measures denatured proteins while the ELISA attempts to detect the native cystatin E/M, possibly in complex with the interacting proteases. It is also possible that the amount of cystatin E/M in the media collected from the melanoma lines is below the limit of detection in the ELISA assay, as some cell lines showing strong expression on immunoblotting (e.g. HCT-116, MA11) had low amounts detected by ELISA. Also worth commenting is that different antibodies are utilized in the two assays, and differences in their affinity for the various forms of cystatin E/M might be possible. The polyclonal antiserum used in the ELISA has, however, been demonstrated to recognize both the glycosylated and non-glycosylated forms of cystatin E/M (data on shown), making this explanation less likely. Nevertheless, there was a strong correlation between the ELISA results for cystatin E/M and the non-glycosylated form observed by immunoblotting.” In the revised manuscript, the last sentence has been modified to specify that the correlation was statistically significant as also reported in the result section (page 16).
5) Although the secreted cystatins clearly inhibit the enzymatic activity of added legumain and
cathepsin B (so they are functional inhibitors), this does not mean that their cellular loss
activates the two proteases.

Author’s comments: Again, it is hard to understand what the reviewer means by this comment.
We do not state that secretion (“loss”) of cystatins activate the cellular proteases. In contrast,
high cellular production of cystain E/M was observed as high concentration of the inhibitor
present in media (as expected for a secretory cystatin), as well as low cellular protease activity.
For further details, see comment 6.

6) In fact in each cell line where cystatin E/M is lost/secreted (MCC13, 70, 57 add 72) (Fig.
1A), the levels of endogenous legumain and cathepsin B drop (for unknown reasons) (Fig.
2). This means that the activities of these two proteases do not increase (what should be
required for sensitizing towards metastasis) but probably even decrease. Endogenous
legumain and cathepsin activities are not measured.

Author’s comments: As stated in the original manuscript (M&M, results, discussion), we have
measured legumain and cathepsin B activities in all cells lysates included. In addition, we have
now included cathepsin L-activity. Figure 1A shows cystatin E/M secretion (“loss”) to the cell
media and figure 2 shows legumain, cathepsin B and L expressions in the corresponding cell
lysates. We also reported that linear regression analysis revealed a positive correlation
between cellular legumain activity and expression of the putative active 36 kD form in cell
lysates. The individual legumain activity data are shown below but not included as a figure in
the manuscript. These results are hopefully clearer in the revised manuscript.

Also, the profiles of inhibitors and proteases of MCC13, 70, 57 and 72 do correlate with
the observed interplay of cystatin E/M and legumain after CST6-transfection (see point 3).
We acknowledge the constructive comments and hope that the 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