Reviewer's report

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

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Reviewer: Christoph Borner

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The present MS is based on previous findings that lysosomal cysteine proteases such as legumain and cathepsin B can facilitate the invasion of a variety of tumor types. Particularly in breast cancer, it was proposed that a loss of the cysteine protease inhibitor cystatin E/M might be responsible for this effect. The present study extends this principle to the skin, i.e. to the invasion of malignant melanoma cells. The authors used a series of non-melanoma, established melanoma, primary melanoma and metastatic melanoma cell lines to measure the presence of cystatin E/M and C in the cell media by immunoblotting and ELISA. In addition, legumain and cathepsin B expressions and activites were measured in the cell lysates by immunoblotting and specific enzymatic assays. Two out of five primary and two out of six metastatic melanoma cells lines showed increased secretion of cystatin E/M in a mature, highly glycosylated form (17 kD). When two melanoma cell lines lacking cystatin E/M secretion were transfected with cystatin E/M they showed increased invasion/migration by the transwell Matrigel assay.

Unfortunately, the data obtained from cystatin E/M immunoblotting and ELISA did not match, the right control cell lines, such as normal melanocytes or keratinocytes were not included in the study, the amount of cellular (not secreted) cystatins was not measured and the decrease of cellular cystatins was not associated with increased legumain or cathepsin B levels or activities, but rather a decreased expression of both proteases. Thus, the conclusion from this MS is rather iffy and inconsistent, and therefore this paper does neither support the previous data from breast cancer cells nor provide a better understanding about the causal link between cathepsin expression/activity and metastatic potential.

Major points:

A correlation between increased lysosomal cysteine protease expression and activities and metastasis has already been reported in several previous studies. Thus, the study here is not novel, except when we additionally consider the regulation of cathepsin and legumain activities by their inhibitors (cystatin E/M). However, in order to make this principle convincing, more experiments are required than the ones shown here.

1) Only secreted cystatin E/M and C levels are examined, but not how much of the inhibitors remain inside the cells.
2) Control cell lines (non-cancerous skin cells) are missing

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.

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.

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.

6) In fact in each cell line where cystatin E/M is lost/secreted (MCC13, 70, 57 and 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.

The only convincing data in this MS are those which show that cystatin E/M overexpressing cells migrate faster in the transwell Matrigel assay. This is however expected from previous report and does not advance the field much. What is needed is a clear causal relationship between cystatin expression and legumain and cathepsin B activities in the various primary and metastatic melanoma cell lines.

**Level of interest:** An article of limited interest

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.