Author’s response to reviews

Title: Impact of uPA/PAI-1 and disseminated cytokeratin-positive cells in breast cancer

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Author’s response to reviews:

Dear Editor Anne Menard:

Please find attached our revised manuscript:

“Impact of uPA/PAI-1 and disseminated cytokeratin-positive cells in breast cancer”.

We very much appreciate the efforts of the editor and reviewers spending the time to read and commenting on our manuscript. We performed the requested additional calculation and revised the manuscript completely according to yours and the reviewer’s comments. A point by point answer is provided at the end of this letter.

We hope that our manuscript is now suitable for publication in BMC Cancer.

Sincerely
Bruno Märkl, M.D.

Point-by-point response

Editor Comments:

1. Please note that all manuscripts must contain all the following sections under the heading 'Declarations'. The Declarations should follow the Conclusions section, and be before the References.

Ethics approval and consent to participate

Consent for publication

Availability of data and material

Competing interests

Funding

Authors' contributions

Acknowledgements

Answer:

We followed these instructions thoroughly.

Serenella Eppenberger-Castori, Ph.D. (Reviewer 1):

uPA and PAI-1 are very well established biomarkers in breast cancer. The idea of this study to further investigate if the presence of these tumor aggressiveness markers correlates with disseminated disease in the bone marrow via detection of cytokeratin-positive cells (dCK+) is valid.

The reported associations of the uPA and PAI-1 expression levels with HR-, HER2- status, tumor grading and patients' overall survival are in accordance with data already published with larger collective, showing that this retrospective cohort as well as the detection of this protease and its inhibitor are representative.

Answer:
We are thankful to Ph.D. Eppenberger-Castori and appreciate all her efforts for reviewing our manuscript and allowing us to revise our work.

The reported association between dCK+ cells with the occurrence of lymph node metastases (P < 0.005) is reported to be highly significant but dCK+ lack any prognostic impact as single marker. No association between dCK+ and PAI-1 is observed. The one with respect to uPA positivity is only marginally significantly: P = 0.028.

This is certainly due to the fact, as also discussed by the authors, that the number of cases in the subsets is very limited.

The p-values corresponding to the survival curves depicted in figures 2B and 3 D refer to the overall difference between the curves and not between the triple plus and the two plus. Therefore, the authors should perform a statistical analysis comparing these two curves. Only if this would be significant the author could affirm the added synergistic values of dCK+.

Taking all together the message of this manuscript in the present form is underpowered and doesn't allow any clear conclusion.

Answer:

According to the reviewer’s recommendation, we performed calculations for the pairwise comparison of the different subgroups of our collective. Additionally, we also integrated the subgroup of triple-negative cases in Figure 2B and provided these data on Page 7:

However, a significant difference, was found when comparing triple negative cases versus PAI-1-negative versus PAI-1-positive cases with uPA- and PAI-1- positivity (double positive) versus uPA-, PAI-1- and dCK+ cell positivity (triple-positive) (P = 0.022 BHST 0.01) with mean survival times of 127 months (CI: 116 – 138 months), 126 months (CI: 117 – 135 months) (P = 0.045 BHST 0.01), 107 months (CI: 98 – 116 months), 115 months (CI: 108 – 122 months), and 90 months (CI: 75 – 105 months) (Figure 2B). Pairwise Multiple Comparison Procedures (Holm-Sidak method) revealed significant differences between triple positive cases and PAI-negative cases (P = 0.014) and triple negative cases (P = 0.036). All other combinations were not significant

Moreover, Page 8:

Pairwise multiple comparison procedures (Holm-Sidak method) revealed only a significant difference between the groups triple positive and one or none positive marker (P = 0.001), all other combinations were not significantly different.

Additionally, we corrected an error on Page 8:
were highly significant prognostic factors for overall survival (uPA: positive vs negative 91 months (CI: 78 – 103 months) vs 119 months (CI: 108 – 129 months); P = 0.006 (BHST 0.017). We also changed the headings of Figure 3 from “nodal” to “node”.

We agree with the reviewer that an additive effect of dCK+ cells cannot be proven by our study a weakened our statement in the discussion part on Page 10:

However, we possibly identified an additive effect when dCK+ cells were identified in cases with uPA/PAI-1 positivity. This finding must be considered with caution because the difference between double and triple positive cases lacked significance.

Galatea Kallergi, PhD (Reviewer 2):

The manuscript entitled "Impact of uPA/PAI-1 and disseminated cytokeratin-positive cells in breast cancer" is interesting. The authors tried to find combination of biomarkers that could be prognostic factors for breast cancer patients, however there are some questions that need to be addressed.

Answer:

We are very thankful to Professor Kallergi and appreciate all her efforts for reviewing our manuscript and allowing us to revise our work.

Why the authors have used an antibody targeting only cytokeratin 18 to detect DTCs while most of the studies for Circulating Tumor Cells and Disseminated Tumor Cells are using either CK19 or pan Cytokeratins antibodies. It is possible that they have lost some positive cases.

Answer:

We agree with the reviewer that currently methods available showing a higher sensitivity compared to CK18 based analyses. However, this is a retrospective study that comprises cases between 1999 and 2010. The technique was well established and our positivity rate of 23% indicates an appropriate sensitivity. We mentioned this argumentation now in the discussion part on Page 9:

The technique of immunohistochemistry (CK18) based was well established in our laboratory and revealed reliable results [9, 19]. Using the same technique in colon specimens, we also detected positive cells in benign cases with diverticulitis [20]. Therefore, we think it is appropriate to avoid referring to these cells as tumor cells instead of dCK+ cells. Twenty-three percent of the cases in our collective were dCK+. This is a slightly lower rate compared to the pooled analysis including 4,703 patients with a positivity rate of 30.6%. The positivity rate in this study, however, differed considerably between the contributing centers from 12.4% to 43.9%.
The authors have shown that the presence of DTCs concomitantly with uPA and PAI-1 is associated with poor prognosis. It will be interesting to examine the potential co-expression of these molecules in patients' DTCs. They could probably do this indicatively in some cases. This fact could explain why the triple positivity is a poor prognostic factor.

Answer:

We absolutely agree with the reviewer on this point. Unfortunately, we have no chance to perform additional analyses in these cases because the smears are between 10 and 20 years old and no unstained slides are available. We know mention this clear limitation of our work in the discussion part on Page 11:

A further limitation is the missing availability of further biomaterial for additional analyses like co-expression of different markers on dCK+ cells. Despite these limitations this study could serve as a basis of further investigations employing modern detection methods combined with single cell analyses.

It is strange that DTCs are not prognostic in any group of patients in this study, since many other studies in the past have shown that DTCs could be a poor prognostic factor. Have the authors checked OS or PFS with Cox Regression regarding DTCs numbers in all different groups?

How the authors explain the fact that although the DTCs are not important for survival in this study the triple positivity is an independent prognostic factor.

Answer:

We performed several subgroup analyses regarding the prognostic significance of dCK+ cells. None delivered a difference dependent on the occurrence of dCK+. We propose the existence of a synergistic effect between proteases and dCKs+ cells. The proteases facilitate the detachment of tumor cells and their migration.

Moreover, they protect tumor cells from immune surveillance. Angiopoesis is enhanced as well as proliferation. This could explain why the simultaneous existence of tumor cells and activation of the plasmin-plasminogen system was the strongest prognostic factor in our study. We discussed that on Page 10 bottom.

Further corrections

Additionally, to the changes made based on the reviewer’s comments, we had to perform some further revisions:

1. Due to the change of the status of our hospital, we changed the affiliations of BM, MK, EJ, RH, TJ, GeS from Klinikum Augsburg to Universitätsklinikum Augsburg.

2. On Page 8 there was an error OS for uPA positive is not 105 but 91 months.
3. The headings of the figures 2 and 3 were corrected from “nodal” to “node”.