Author’s response to reviews

Title: Soluble EpCAM levels in ascites correlate with positive cytology and inhibit catumaxomab activity in vitro

Authors:

Andreas Seeber (andreas.seeber@uki.at)
Agnieszka Martowicz (agnieszka.martowicz@ki.se)
Gilbert Spizzo (gilbert.spizzo@i-med.ac.at)
Thomas Buratti (thomas.buratti@asbmeran-o.it)
Peter Obrist (peter.obrist@tyrolpath.at)
Dominik Fong (dominic.fong@i-med.ac.at)
Guenther Gastl (guenther.gastl@i-med.ac.at)
Gerold Untergasser (gerold.untergasser@i-med.ac.at)

Version: 2 Date: 31 January 2015

Author’s response to reviews: see over
Dear Editors,

Please find enclosed our revised manuscript.

We extensively revised the manuscript according to suggestions of both referees and included additional experiments that both referees requested in particular on tumor cells. We believe that our work now fits the scope of BMC Cancer and is of interest for a broad and diverse audience.

Sincerely Yours,

Gerold Untergasser, PhD
Reviewer #1:

(1) Apart from this finding the cancer-relevant part of the study, however, is weak. For the ascites measurements, only small patient cohorts were used where positive cytology and sEpCAM only infrequently occurred in the aspirates.

Ad 1) We agree with the reviewer that the cancer part is weak and included new data on sEpCAM levels in different human cancer cell lines (new Figure 4C), serum samples (new table 4) and performed ADCC studies also with EpCAM positive human cancer cells (new Figure 7). Since not all tumors (in particular tumor cells with mesenchymal phenotype after EMT) express EpCAM we do not monitor sEpCAM in all cytology positive ascites. This fact is discussed on page 15. The limitation to small cohorts in this study is due to the fact that we used only patients from one center (Merano, South Tyrol, Italy) that were sampled the last 5 years.

(2) Moreover, EpCAM levels were often below the detection threshold of the ELISA system and the background noise by inflammatory degenerative events like liver remodeling was comparatively high.

Ad 2) Background levels due to massive cell death and remodeling in liver are discussed on page 15/16. We cannot exclude that 4 out of 31 patients had strong remodeling processes leading to massive cell death and release of EpCAM from liver stem cells.

(3) It also remains unclear if all measured sEpCAM was produced by cleavage and shedding from the surface of viable cells or resulted from massive tumor cell death in the ascites.

Ad 3) Measured sEpCAM can be cleaved EpEX or EpCAM on surface of exosomes released by tumor cells as discussed on page 16. Tumor cells that undergo necrosis release many membrane vesicles and thus, can contribute to the high levels of sEpCAM in ascites. Thus, as mentioned by the referee, our ELISA cannot discriminate between EpEX produced by proliferative signaling and shedding from tumor cells or sEpCAM from exosomes of necrotic/apoptotic tumor cells. We include new data about sEpCAM levels in proliferating colorectal carcinoma cells and cells undergoing cell death due to apoptosis or necrosis (new Figure 4C).

(4) It is unclear what the authors mean with the prognostic value of EpCAM in ascites.

Ad 4) Diagnosis of peritoneal carcinomatosis is based on positive cytology in ascites. Analysis of sEpCAM helps to get additional information next to cytology about the presence of EpCAM high tumor cells in ascites. Cytology alone has the limitation of low sensitivity to detect tumor cells. Indeed, we could detect some patients that were sEpCAM positive, but negative in cytology. The combination of both methods, sEpCAM as biomarker and cytology is more powerful in the detection of peritoneal carcinomatosis. These conclusions and limitations are discussed on page 15.
Surprisingly, the amount of sEpCAM produced by tumor cell lines (HEK 293) in vitro was far below the levels required for catumaxomab inhibition.

Ad 5) Based on this good comment we measured sEpCAM concentrations in vitro in a variety of cancer cell lines positive for EpCAM expression and cancer cells that already displayed EMT (EpCAM low). These data were incorporated in the new Figure 4C. Some epithelial cell lines had levels of sEpCAM sufficient for inhibition of catumaxomab.

The question remains to what extend the efficacy of other EpCAM targeting drug delivery systems will be affected by such competition binding. Only an in vivo tumor xenograft model in mice, e.g. ovarian cancer cells growing i. p., could provide a clue how sEpCAM in biological fluids affects tumor targeting and destruction, and whether its quantification may be useful for therapy decisions.

Ad 6) Based on this comment, we included now ADCC experiments with human tumor cells (HRT-18, colorectal carcinoma cells) and tested ascites with high sEpCAM levels versus negative ones (new figure 7). We found that ascites with high sEpCAM inhibited ADCC of colorectal carcinoma cells. Moreover, preliminary data on ascitis samples showed that patients with high sEpCAM levels had bad clinical outcome under therapy (see attached figure with survival curves). These data support our hypothesis that sEpCAM can be a predictive marker for therapy-response.

Figure: Kaplan-Meier plot of Patients with sEpCAM positive and negative ascites; preliminary data of 66 patients.

Despite proof of concept that sEpCAM can be produced by cytologically positive ascites and may compete anti-EpCAM ligand binding on the cell surface, it would be of interest to measure circulating sEpCAM levels as a marker for tumor targeting and malignant progression also in the blood as this is the more common clinical condition. However, I assume that this will be far below the detection limit.

Inspired by this suggestion we measured also sEpCAM levels in a collective of tumor patients where both, ascites and serum samples were available. We found also sEpCAM levels in peripheral blood of patients with high sEpCAM in ascites. These data are shown in the new table 4.

In the whole Fig. part incl. Fig. legends the name catuxumumab (?) is used. We corrected all figure legends and did a proofreading of the script.
Reviewer #2:

(1) Although manuscript is still weak to meet the authors hypothesis can be improved. To prove that sEpCAM from body fluid neutralizes Catumaxomab and fail to target tumor tissue. Authors need more experiments to convince.

Since the tumor part of the script was week we performed new experiments and included data on sEpCAM levels in a variety of tumor cell lines and under apoptotic/necrotic conditions (Figure 4C). Moreover, we measured ascites samples and serum samples of patients (new table 4) and performed again ADCC assays with catumaxomab on colorectal carcinoma cells with recombinant EpEX and ascites (new Figure 7).

(2) Authors have raised question with data in Figure 6. With previously reported observation that soluble EpCAM can also induce EpIC cleavage from surface EpCAM and activate wnt signaling. In this situation blocking sEpCAM with Catumaxomab mediated therapy can help cancer patients? need to discuss.

We incorporated the interesting data about EpEX and induction of mitotic signaling on breast carcinoma cells (REF. 20) in the introduction part (page 4).

(3) Experiments with HEK293 in Figure 6 cells are convincing, but need to show in at least two cancer cell lines with secreted EpCAM (EpCAM positive cells) or spiked EpCAM (EpCAM negative cells).

We performed the ADCC assays with recombinant EpEX on HRT-18 colorectal carcinoma cells (EpCAM positive) and human diploid fibroblasts (EpCAM negative). As expected catumaxomab did not lyse fibroblasts and catumaxomab-dependent lysis of HRT-18 cells was strongly inhibited by addition of recombinant EpEX (5 ng/mL). These data are shown in new Figure 7.

(4) Need to show Catumaxomab binds sEpCAM in ascites. As Catumaxomab is bi-specific. (is there data cleaved or sEpCAM and surface EpCAM have different binding affinities to Catumaxomob?)

In the revised script we include now data that catumaxomab binds to sEpCAM in ascites. Ascites samples with high sEpCAM were significantly inhibiting catumaxomab-dependent lysis of HRT-18 cells. These data are shown in the new Figure 7. There are no data provided by Fresenius Biotech, the distributor of catumaxomab, about binding affinities of their antibody to EpCAM on cell surface (tetrameric form) and soluble EpEX (monomer).

(5) Cell death assays need to be done with appropriate and independent techniques.

We agree with the referee and performed additional studies on cell death by flow cytometry using an Annexin-V apoptosis kit. Late apoptotic and necrotic HRT-18 cells were stained and quantified after performing ADCC assays with catumaxomab and PBMNCs. These data are shown in the new Figure 7D.

(6) Need to cite literature on clinical trial catumaxomab and their results, and discuss how your observation can help in future trials.
prospective randomized phase II/III trials on catumaxomab are cited (REF 26) and we discuss our data in the context of preliminary data on catumaxomab and sEPCAM levels in ascites and survival of patients.

Figure: Kaplan-Meier plot of Patients with sEpCAM positive and negative ascites; preliminary data of 66 patients (From Berlin, Germany and Innsbruck, Austria).

(7) Reduce discussion part appropriate to manuscript data

We reduced discussion appropriate to manuscript data

(8) Flow cytometry data for dying YFP can be misleading as dead cells fluoresce.

Next to the YFP based quantification of HEK cells after ADCC we performed assays with a Annexin-V kit to quantify viable HRT-18 cells by flow cytometry (Figure 7D).

(9) Data can be added in supplementary Figure 1, Figure 2.

We added new data in table 4 and in Figure 4 (C section) and in the new Figure 7

(10) Appropriate references on clinical trials and EpCAM in body fluid/ serum.

We updated our references