Reviewer's report

Title: Circulating endothelial cells and other angiogenesis factors in pancreatic carcinoma patients receiving gemcitabine chemotherapy

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Reviewer: Christine Brostjan

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The authors have determined the level of circulating endothelial cells (CECs) in the blood of pancreatic cancer patients before and during gemcitabine therapy. CEC concentrations did not change during therapy. Baseline levels of CECs were found to be of (statistically significant) prognostic and predictive value. Patients were grouped according to baseline levels of CECs (below/above mean value). Study participants with high levels of CECs showed shorter progression-free and overall survival. Furthermore, non-responders with progressive disease had significantly higher baseline levels of CECs than responders (partial remission or stable disease) to gemcitabine therapy. Blood concentrations of the cytokines IL-6, IL-8, IL-10, HGF and VEGF were found to correlate with baseline CEC values.

I believe that the article is of interest to the scientific community, despite the fact that our group has recently published a comparable report on the prognostic and predictive potential of CECs in pancreatic cancer patients receiving gemcitabine therapy (Starlinger et al. Neoplasia. 2011 Oct;13:980-90). Since the two studies yielded different results, the authors should comment on the possible reasons, e.g. differences in detection methods of CECs which may account for the discrepancy: Kondo et al. employed the “CellTracks System” by Veridex which involves a bead-based enrichment of CD146+ cells followed by cell labeling for CD45-CD105+DAPI+ cells. In contrast, we performed whole blood staining for CD45-CD31+CD146+ CECs and discriminated between viable and dead (7AAD+) CECs. In contrast to Kondo et al. we found CEC levels to rise significantly during gemcitabine therapy. Post-therapy rather than baseline CEC levels were significantly associated with response and patient survival. It is important to emphasize that the current diversity in detection methods leads to the evaluation of so called “CEC” populations which are not necessarily related and which may exhibit distinct marker potential.

Major Compulsory Revisions
1. There is an alarming frequency of inconsistencies between the results shown in tables and figures and the numbers given in the Results text. List of inconsistencies which need to be corrected:
   a) Table 1: The number of patients listed with stage III, IV or recurrent disease does not add up to the total number of patients in each group (CEC high: 9+8+1 does not equal 12; CEC low: 5+12+2 does not equal 25).
b) Results, “Baseline levels of CEC”: the 95% CI values for PFS as well as for OS have been mixed up between the CEC high and CEC low group, since the respective median values are not within the confidence intervals.

c) Results, “Baseline levels of CEC”: There is a complete inconsistency in the hazard ratios and 95% CI values given for performance status, stage, and CRP level in the text as compared to table 2.

d) Results, “Baseline levels of CEC”: The same holds true for the multivariate Cox analysis, for stage and CRP level.

e) Results, “Association between levels of CECs and blood angiogenic factors”: The text states that HGF baseline levels were significantly higher in patients with PD as opposed to those with PR or SD. In contrast, figure 3 shows a p-value > 0.05 for HGF, indicating that there is no statistically significant difference between groups!

f) Results, “Changes in CEC levels during treatment”: The last line states that CEC changes during treatment were not statistically associated with tumor response but gives a significant P-value < 0.05?!

2. The article holds a major misinterpretation of results: The authors have observed significant correlations between CEC levels and blood concentrations of various cytokines (IL-6, IL-8, IL-10, HGF and VEGF). They repeatedly imply that this correlation indicates a causal connection between these cytokines and circulating CEC levels. This is a gross overstatement. A correlation between blood parameters is no proof for a causal relationship. The authors would need to carefully rephrase their statements and reduce the emphasis on this topic in the discussion section. It is likely that both, cytokines and CEC blood levels are connected to a higher blood vessel turn over in more aggressive tumors, but a causal relationship cannot be concluded from the data presented.

Minor Essential Revisions

1. The Methods section lacks some important information:

   a) Dosage, schedule and duration of gemcitabine treatment?

   b) CECs were determined for which blood volume: 4 mL?

   c) Did the CEC analysis include a measure of dead cells?

   d) What was the intraassay variation (standard deviation in % of mean) with respect to the duplicate measurements of samples?

   e) How was blood processed for plasma preparation? The mode of plasma preparation greatly impacts the reliability of VEGF measurements (Starlinger et al. Dis Markers. 2011 Jan 1;31:55-65).

   f) Cytokines were assayed in a „subgroup of patients and controls“ according to the authors: Please state the number of patients and controls included in cytokine analysis to be able to judge the statistical impact of correlation with CEC levels. The fact that 5 out of 7 investigated cytokines showed a highly significant correlation with CEC levels (P < 0.01 and k = 0.4 to 0.8) is highly unexpected. Furthermore, why did the authors chose Pearson instead of Spearman
(non-parametric) for the evaluation of correlation?
g) The abstract mentions cytokine evaluation by ELISA; the methods section does not explain which cytokines were evaluated by ELISA as opposed to bead technology.

2. Based on the small number of patients and the difference between mean (166 cells/4 mL) and median (66 cells/4 mL) level, a normal distribution of CEC values seems unlikely. Nevertheless, the authors chose the mean (as opposed to the median) for a cut-off value. They should comment on this decision, especially since they apply non-parametric statistical tests (Mann-Whitney U test).

3. The authors report that CEC levels measured 28 days after start of gemcitabine therapy were not significantly different from baseline CEC values. In our own study (Starlinger et al. Neoplasia. 2011 Oct;13:980-90) we observed that CEC values substantially increase when measured within 1 week of the last gemcitabine administration. However, after a treatment gap of 2 weeks, CEC levels have recovered. It would therefore be important to know, whether the authors measured CECs within one week of gemcitabine administration.

4. Results, “Baseline levels of CEC”: The authors report that CEC levels of pancreatic cancer patients were higher than those of healthy volunteers, but they do not state whether this difference was statistically significant. They should give a P-value.

5. Discussion: The authors claim that their “findings agree with those of a previous study (by Ko AH et al. 2010) that found that the baseline level of CEC was inversely associated with overall survival” in pancreatic cancer patients. However, the authors neglect to mention that Ko et al. compared three methods of CEC detection – including the CellTrack System employed by the authors. Ko et al. report that there was no correlation between methods, and only CECs identified as CD45-CD31+CD34+ by flow cytometry (but not CECs determined with the CellTrack System) showed an inverse correlation with survival. Thus, the authors should carefully rephrase their statement.

6. Discussion: The authors further mention a previous study by their own team on CEC levels in non-small cell lung cancer patients (Kawaishi M. et al. 2009) where CECs were found to decrease following chemotherapy. However, the same study reports that low baseline CEC levels were associated with a poor response (PD) to treatment. This is the exact opposite of what was now found for pancreatic cancer patients receiving gemcitabine: non-responders (PD) showed high baseline CEC levels. The authors fail to comment on this discrepancy.

Discretionary Revisions

1. Overall the article is well-written, with a logical presentation of aims, figures and tables. There are minor typographical errors such as:
   a) The in-text numbering of references should read [19-23] instead of [19][20][21][22][23].
   b) PDGF-BB: B does not stand for beta, but for factor B
   c) ECOG: Eastern (not Easter) Cooperative Oncology Group
2. It would be informative to include the number of patients and healthy controls in the abstract.

3. Methods, “CEC Enumeration”: There are two references (3,4) which are misplaced, i.e., do not refer to the required articles.

4. The authors claim that this “is the first study to show the clinical importance of CEC levels as a prognostic factor in advanced pancreatic carcinoma treated with gemcitabine-based chemotherapy”. Considering our own study which has been published in Oct. 2011 (Starlinger et al. Neoplasia. 2011 Oct;13:980-90) this statement does not hold true.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** Yes, and I have assessed the statistics in my report.

**Declaration of competing interests:**

I declare that I have no competing interests.