Reviewer's report

Title: CD133 expression in chemo-resistant Ewing sarcoma cells

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on the manuscript entitled: "CD133 expression in chemo-resistant Ewing sarcoma cells" by Jiang X et al. (Corresponding author: Elizabeth R. Lawlor)

The manuscript by Jiang et al. investigated the expression of CD133 in Ewing sarcoma family tumors (ESFT) and cell lines and their association with drug resistance. The study was designed to evaluate the relative importance of CD133 as a putative marker of tumor-initiating cells in ESFT. Despite the recent identification of CD133 as a putative stem cell marker in this tumor type (Suva et al. Cancer Res. 2009;69:OF1-6), PROM1 expression was either absent or very low in most tumors. Only in a small subgroup of patients CD133 was highly overexpressed. In a RT-PCR and western blot-based screen of ESFT cell lines the expression of CD133 was detectable in 5/9 lines albeit with a heterogeneous pattern as revealed by immunostaining and flow cytometry. Importantly, only in one cell line (STA-ET-8.2) differences in tumor-specific properties between CD133+ and CD133- cell fractions were found that were compatible with the concept of cellular hierarchy, e.g. where colony-forming, tumorigenic, and chemoresistant CD133+ cells gave rise to less tumorigenic and chemosensitive progeny. The authors conclude that significant heterogeneity exists between both primary ESFTs and established cell lines and that CD133 is not a suitable marker with which to identify chemo-resistant/tumor-initiating cells.

This study is straightforward in its design, with the choice of methods being appropriate and their performance technically sound. It uncovers that CD133 is not an universal marker for tumor-initiating cells in this tumor entity. A strength of this study is that a much larger cohort (48 samples) has been analysed than in the work of Suva et al (Cancer Res. 2009;69:OF1-6). The data from Jiang et al clearly disregard CD133 as an universal marker for chemoresistant tumor-initiating cells and therapeutic target for this tumor entity. Despite the convincing data several issues need to addressed or clarified by the authors before this work merits publication in BMC Cancer.

I. Major Compulsory Revisions:

1. The authors used CD133/2 for FACS and CD133/1 for magnetic bead labelling and separation preceding FACS. Is it mandatory to use two different antibodies
and does the preselection with CD133/1 allow for a direct comparison of the FACS-sorted populations generated with CD133/2?

Is the CD133/2 antibody from Miltenyi the same clone (293C3) that was employed by Suva et al. (Cancer Res. 2009;69:OF1-6)? The authors must resolve these issues.

2. Two transcripts of different length have been described to encode CD133. Do the PCR primers which the authors used detect both mRNA transcripts? Why were some samples completely negative in the RT-PCR shown in Fig. 2A (even a few cells would be expected to give a positive signal in a standard RT-PCR assay). For comparison: Suva et al reported 8/8 patients to be positive by (presumably less sensitive) protein-based detection methods like flow cytometry/immunocytochemistry! Is this due to a sensitivity problem of the PCR method applied in this study?

3. The PCR data were normalized with only one housekeeping gene (GAPDH). Ideally, data for PROM1 should be normalized with at least two HKGs to minimize possible variations among samples.

II. Minor Essential Revisions:

1. The labelling of the ordinate and abscissa in Fig. 1 panel A needs to be changed (ordinate: Expression, abscissa: Number of tumors). According to the first paragraph on page 10 (Results) and the corresponding legend this panel is supposed to show the expression of 48 tumor specimens, however I see only 14. What does the numbering on the abscissa refer to? The 11 cases in which PROM1 was readily detected? This needs to be rectified.

2. Page 5, first paragraph, last sentence: the last part of this sentence should be changed to: ...established cell lines and we conclude that CD133 is inconsistent as a marker of ...

3. Figure 6: Do results were similar when the cells were exposed to the cytotoxic drug combination for shorter periods of time?

To strengthen their conclusions on the role of CD133 expression for chemoresistance in the STA-ET-8.2 cells, corresponding results from one of the other ESFT cell lines should be depicted as part of panel A.

4. Page 11, first paragraph: Figure 1B is incorrect as these data (frequency of CD133+ cells in tumor tissue section) are shown in panel C. "Fig. 1C" should be moved either behind "frequency of CD133+ cells" or behind "induction chemotherapy" to precede the conclusion drawn here. The term "data not shown" should be inserted behind "the level of PROM1 (transcripts)" because mRNA data were not depicted in this figure.

5. Page 14, second paragraph: Did the authors, as a control, try to expand in culture clonally derived spheres also from single CD133- cells to see whether they can give rise (spontaneously) to CD133+ progeny?

6. Page 17: Since the process of FACS-sorting negatively affected the tumor-initiating capacity of STA-ET-8.2 cells, did the authors repeated this animal
experiment with cells purified with magnetic beads?

III. Discretionary Revisions

1. Abstract (first sentence on page 3): This sentence is incomplete. It should be stated for what features the authors compared the CD133+ and CD133- negative cells (and have found no differences).

Please note: The pages of this manuscript were not numbered! I have numbered the front page containing the title and authors as #1.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

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