Reviewer's report

Title: CD133 expression in chemo-resistant Ewing sarcoma cells

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Reviewer: Nicolo Riggi

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REVIEW

In this work Jiang et al assess the expression of the cancer stem cell-associated surface marker CD133 in 48 primary human Ewing sarcoma samples and 9 cell lines using RT-PCR, Quantitative Real-Time PCR, Western Blot and Immunohistochemical analysis. The authors report low expression of CD133 in 44/48 primary tumors and strong variability of its expression on the surface of the nine Ewing sarcoma cell lines. However they found that the STA-ET-8.2 cell line retains a cellular hierarchy in which CD133 positive cells display increased tumorigenic and chemo-resistant potential compared to their CD133 negative counterparts. Based on these observations the authors conclude that CD133 is an inconsistent marker of chemo-resistant tumor initiating cells.

Major Compulsory Revisions

The work is well written, the experiments and methods are clearly described and the figures appropriately represent the corresponding text in the results section. However the study suffers from several weaknesses.

1- First, the novelty that the manuscript provides to the Ewing’s sarcoma cancer stem cell field is limited, since a recent article, published in Cancer Research in 2009 (Ref 16), has already identified the existence of a cancer stem cell population in Ewing sarcoma using freshly isolated primary tumor specimens. The novelty of the present work is therefore restricted to the discovery of a Ewing sarcoma cell line containing a CD133 positive population of cells that seems to retain the cancer stem cell hierarchy where the CD133 positive cell fraction displays increased chemo-resistance.

2- The second major problem is the use of inappropriate methods to test the expression of CD133 in order to conclude that it is an ‘inconsistent’ marker of chemoresistant tumor-initiating cells. Indeed, only a specific glycosylated epitope of CD133 is related to stem cells and cancer stem cell biology in all published studies thus far, and this epitope is recognized only by the AC133 monoclonal antibody (Miltenyi). In their work, the authors use RT-PCR and Quantitative Real-Time PCR to assess the expression of CD133 in primary tumors and cell lines (Figure 1A, 1B and 2A), but it is obvious that the results obtained cannot give any significant information regarding the expression of the stem cell-related epitope of CD133, or the presence of cancer stem cells in the tested samples. Moreover, the Western Blot and Immunohistochemical analysis (Figure 1C, 2A...
and 2B) performed by the authors in order to test the expression of CD133 in only two primary tumors and the different cell lines, are performed using Santa-Cruz and Abcam polyclonal antibodies, instead of the antibody recognizing the glycosylated, stem cell-related, AC133 epitope (Miltenyi). This approach hampers any identification or quantification of CD133-positive cancer stem cells in all the samples tested, and the final conclusions of the work based on these experiments are therefore flawed. The only possibility to investigate a broad panel of primary tumors for the presence of CD133 positive cancer stem cells is immunohistochemical analysis of frozen sections and the concomitant isolation of tumor cells from fresh surgical specimens of Ewing sarcoma using the AC133 antibody, followed by assessment of the tumorigenic potential displayed by the two cellular fractions. Without these experiments, the authors should at least reformulate their conclusions about the presence and percentage of chemo-resistant cancer stem cells in primary Ewing sarcoma and clearly discuss these limitations in the abstract and the discussion sections.

3- For the characterization of the STA-ET-8.2 cell line, the authors use the AC133 antibody to assess the percentage of CD133 positive cells, and to further sort the two fractions by FACS analysis, prior to soft agar, tumorigenic and chemo-resistance assays. The results obtained using the STA-ET-8.2 cell line are therefore interesting since they offer the possibility to use a cell line to further investigate the biological properties of the Ewing sarcoma cancer stem cells. However, the tumorigenic assay should be improved: the authors use 5.106 tumor cells to test the difference in tumorigenic potential between the unsorted, CD133 positive and CD133 negative populations, and find a significant difference between the two sorted fractions but also a surprising decrease in tumorigenicity compared to the unsorted cells. The number of cells used for this experiment is very high and begs the question what is the minimal amount of cells needed to obtain a tumor from the unsorted cells? It would be interesting to repeat the experiments using a more permissive animal model (such as NOD-SCID Common-g KO), sorting the cells using only magnetic microbeads (that the authors use to enrich CD133 cells prior to FACS analysis), and performing the subcutaneous or orthotopic injections using limiting dilutions of the cells, to determine the minimal amount of CD133 positive or unsorted cells needed to obtain tumor formation.

In the same result section, the comparison made by the authors between the percentage of CD133 positive cells in the tumor xenografts and in the negative fraction prior to injection into NOD-SCID mice is arbitrary, since the former assessment is performed using a polyclonal antibody and the latter using the monoclonal AC133 antibody that recognizes only the cellular subpopulation expressing the stem cell-related glycosylated epitope of the CD133 protein.

4- Finally, in the determination of the chemo-resistance it would be important to test the sensitivity of the CD133 positive and negative cells to the different chemotherapeutic drugs alone and in different combinations, instead of testing only the three drugs together.

Minor Essential Revisions
1- In the result section there is an inversion between Figure 1B and 1C in the text.

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

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

'I declare that I have no competing interests'