Reviewer's report

Title: CD133 expression is not an independent prognostic factor in stage II and III colorectal cancer but may predict the better outcome in patients with adjuvant therapy

Version: 5 Date: 12 November 2012

Reviewer: CLAUDIU MARGARITESCU

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Abbreviations used must be explained at their first appearance in the main body of the manuscript (not only in the abstract), for example: CSC, CRC, IHC, HR.

-Materials and Methods.

1) As it regards the histologic differentiation, we suggest to the authors to separate the poor differentiated adenocarcinomas from mucinous variant, they are different types of colorectal adenocarcinoma with different clinico-morphological characteristics.

2) Immunohistochemical analysis.

a) In what it regards the used CD133 antibody, the authors must specify all its characteristics (species, clone, the exact epitope detected, its cellular location), even more the specified manufacturing company code (Abcam has withdrawn some anti-CD133 antibody from sale). Also, the IHC protocol does not specify the producer of the secondary amplification system and DAB.

A series of reports had shown that we should be very careful when using this surface marker for CSC identification on solid tumors. For example, the two most used CD133 monoclonal antibodies are raised against the AC133-and respectively AC141-CD133 epitopes and thus bind only to glycosylated sequences of CD133 with the possibility of false negative results when nonglycosylated CD133 is also to be detected (Corbeil et al., 2000, Miraglia et al, 1997).

Another source of false positive results could be the cross-reactivity of anti-AC133 or AC141 mAbs with other glycosylated epitopes existing on extracellular molecules (Biddingmaier et al., 2008). In addition, a cross-reactivity of AC141 mAb with cytokeratin 18 has been shown, especially in fixed tissue or cells with damaged membranes (Pötgens et al., 2002). More than that, it was shown that AC133 and AC141 epitopes can be down-regulated independently from the CD133 protein or mRNA (Corbeil et al., 2000; Florek et al., 2005), and the tissue distribution of CD133 mRNA was found to be much more widespread than the expression of the AC133 epitope itself (Miraglia et al, 1997).

Additionally, will have to take into account the presence of alternatively spliced CD133 isoforms [Shmelkov et al., 2004; Yu et al., 2002], and that some of them could be lacking the AC133 or AC141 epitopes, this leading to false negative
results.

For all these reasons it is crucially important to specify all the characteristic of the anti-CD133 antibody used in this setup. In addition, the authors must specify how they have taken into account all these inconveniences and how were they resolved.

b) Also we must keep in mind that CD133 labels not only CSCs but also even other stem cells such as: hematopoietic stem cells (Kobari et al., 2001), lymphohematopoietic or lymphoid stem cell (Lönnroth et al., 2012), endothelial progenitor cells (Quirici et al., 2001), mast cells (Rastogi et al., 2008). Therefore, when we use the CD133 for identification of CSCs in colon cancer we must take into account the possibility of labeling a wider cell population. Especially in the cases of poor differentiated adenocarcinomas and in the other histological degree of differentiation of colorectal cancers that associated an abundant inflammatory infiltrate, we should make the differentiation between the CSCs and these non-tumoral cells that could express the CD133 marker. To do so, we must perform double immunohistochemical reactions, for example for CD133+CD34 (hematopoietic stem cells), CD133+CD45 (lymphoid stem cell), CD133+VEGFR2 (endothelial progenitor cells), CD133+tryptase (mast cells). Thus, the IHC assessments off CD133 alone in the tumor specimens will be overstated.

c) The method used to asses the IHC staining of CD133+ CSCs is much more subjective. We strongly recommend to use an image analysis software, with which to make a semiquantitative measurement of IOD (integrated optical density) or to report the results as percentage of CD133+ CSCs from the all tumoral cells that were counted on an appropriately large enough tumoral area to cover an important part of the tumoral volume.

3) RNA Extraction and cDNA synthesis

Taking into account that CD133 can be expressed on normal colo-rectal epithelial cells and even more in other stem cells (such as: hematopoietic stem cells, lymphohematopoietic or lymphoid stem cell, endothelial progenitor cells and mast cells) that are present inside the tumor specimens to obtain correct results it becomes absolutely necessary to carry out a laser microdissection of only CD133+ CSCs. After that, the authors should perform the Quantitative RT-PCR and Bisulfite conversion and pyrosequencing analysis of DNA methylation.

-Discussions

In this manuscript section, the authors must discuss all the limitations regarding this study, including the aspects outlined above.

References:


Quirici N, Soligo D, Caneva L, Servida F, Bossolesco P, Deliliers GL, Differentiation and expansion of endothelial cells from human bone marrow


**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' below.