Author's response to reviews

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

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Version: 6 Date: 13 December 2012

Author's response to reviews: see over
Dear Sirs!

Thank you for the timely review and for the important comments to further improve our manuscript. We are happy that our research work has been thoroughly evaluated and considered acceptable for publication in your journal.

As per the recommendation and valuable comments of the reviewers, we brought some changes and added few paragraphs in the body of the manuscript. All changes and additions appear in red color in the revised version of the manuscript. We kindly reply to the comments of the reviewer as following:

To the honorable reviewer: Mr. CLAUDIU MARGARITESCU

Comment:
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:
Answer:
Rightfully, the above mentioned changes are brought to the main body of the manuscript.

1) Comment:
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.
Answer:
We changed the text in the main body and the table, as per your recommendation (the poorly differentiated adenocarcinomas are separated from mucinous carcinomas in the text and table).

2) Comment on IHC analysis :
   A) Characteristics of the antibody used (species, clone, the exact epitope detected, its cellular location) and the inconveniences with IHC staining representativeness and method for evaluation of IHC staining.
   Answer:
   As for the evaluation of immunohistochemistry, we selected one of the most representative and adequate sections from each patient for immunohistochemistry. The characteristics of the primary antibody were as following:

   CD133/1 (AC133) pure, Human, Miltenyi Biotec, MACS, CA, USA. Lot no.: 5111014074

   The cellular localization of this epitope is plasma membrane protrusions. The antibody was used in a dilution of 1:100. The ultraView Universal DAB Detection Kit was used for IHC staining.
The chemicals used in the IHC staining process, such as DAB and CC #2 were produced by Ventana medical system, Roche, Tucson, USA and the auto-stainer was BenchmarkXT (Ventana medical system, Roche, Tucson, USA). The information regarding the antibody and other chemicals used in the process of IHC staining are added to the manuscript (under material and method part; IHC analysis; page 7)

It needs mentioning that we at first tested antibody from Abcam, however, due to the further unavailability of antibody (because the company did not produce CD133 antibody anymore), we had to test primary antibodies from other company. Therefore, we tried testing and using primary antibody from the MACS, Miltenyi Biotec, CA, USA. We are extremely sorry for the mistake in writing of the company name of the antibody and we greatly appreciate the comments that brought the mistake to our sight.

Previous studies to which you have pointed in your comments indicated that the alteration of IHC detection of CD133 (AC133 or AC141 epitope) might be due to the changes in the glycosylation of the epitopes, however, recently, it was shown that although the mRNA or protein level of CD133 detected by PCR do not coincide with IHC detection by monoclonal antibodies, the glycosylation of the epitopes or the existence of spliced variant of CD133 has little or no impact of the detection of the epitopes by monoclonal antibody AC133 (Kemper et al.2010). This matter is mentioned in the revised version of the manuscript on page 18).

B) The concern of overstating the results of CD133 IHC staining of real tumor cells with those of non-tumoral cells such as endothelial progenitor cells, or mast cells (double staining):

Answer:

It is herewith stated that no non-epithelial cells were positive in any of the cases in our study including stromal cells, mast cells, etc…. And the use of double staining with other antibodies to target the non-epithelial different lineages were not considered necessary because it is not difficult for experienced pathology experts to differentiate the epithelial malignant cells from the inflammatory cells, stromal cells, mast cells, and endothelial progenitor cells in the tissue sample by matching the IHC stained slides with H&E stained slides based on characteristics of malignant cells such as high N/C ratio and hyperchromasia. However, we strongly believe that it is a good idea and will encourage this consideration in the future work. And at last, we believe (in the case of IHC interpretation) the CD133 positivity in cancerous tissue is far much higher than to
consider double staining for the detection of/differentiation from (few if any) non-epithelial cells that may be positive for CD133.

C) The method used to assess the IHC staining of CD133+ CSCs is much more subjective. We strongly recommend to use image analysis software:

Answer:

Since the existence of CD133 staining were observed on the secretions and shed tumor cells in the lumen of the gland, the use of image analysis software was limited and it was unable to thoroughly represent the immunoreactivity (due to false positivity of the tumor area because of the secretions.

So we resorted to the usage of pathology experts to accurately define the positivity and to distinguish the true positivity of the luminal side of the glands from the secretions inside the gland. The IHC staining results were evaluated independently by two pathologists blinded to the patients’ clinical and pathologic information. Discrepancies between the pathologists were resolved by consensus. As the positivity to this antibody in tumoral tissue was not homogenous, and additionally as we used the whole-tissue-block-mounted slides for IHC staining (not tissue micro-array), the semiquantitative method was used to evaluate the IHC staining. The whole area of the tumor (the H&E slides were used for comparison) in each slide was counted as 100%. The
area of the tumor that was immunoreactive for CD133 was calculated as percentage subtracted from the total volume of tumor. For example, if 20% of the whole tumor cells were positive for CD133, it was counted as 20%. Then, for the application of the IHC results, we divided the IHC results into four groups. Group 1 (no single cell positive for CD133), Group 2 (less than 10% of the whole tumor cells were positive for CD133), Group 3 (11-50% of the whole tumor cells were positive for CD133) and Group 4 (51-100% of the tumor cells were positive for CD133). Group 3 and 4 were counted as CD133 positive. The interpretation of IHC results have already been described in the material and method and discussion parts (pages 7, 8, 14 & 15).

3) Comment

RNA Extraction and cDNA synthesis:

Answer:

We sampled tumor area from the surgically resected specimen for RNA extraction and cDNA synthesis. As we did immunohistochemistry to the same cases and matched the results of the IHC staining with those of mRNA expression, we found a significantly direct correlation between the mRNA expression and the IHC expression levels (see result page 12 and Table 4). Additionally, the great advantage of IHC staining is the ability of morphologic correlation. And using IHC staining, we observed no immunoreactivity for CD133 in the non-tumoral area except for rare cells in the crypt base of non-neoplastic glands (Please see the following photo)
And since there was a positive correlation between the IHC and mRNA levels (in the tumor component), we assume that with proper evaluation of the IHC results and with the microdissection of only tumoral area, it was possible to draw a fair conclusion over the results of our experiments. The microdissection of only the CD133 positive cells (after IHC) for mRNA extraction has a big limitation. And that’s because CD133 is not a nuclear or cytoplasmic staining on one hand and the luminal staining of CD133 in moderate to poorly differentiated cases on the other hand make it far too difficult to microdissect few cells (few single cells) among other CD133- cells in a larger group of cases.

Comment:

**Improvement of the discussion part:**

Descriptions about some aspects mentioned above do appear in the revised version of the manuscript with corresponding references (under the discussion section, pages 17, 18, & 19). Additionally, it needs mentioning that there are some commercially available antibodies such as rabbit polyclonal Abs H-284, mouse mAb 32AT1672, and rabbit mAb C24B9 that target unmodified extracellular CD133 epitopes. They have not yet been tested for the purpose of isolating CD133+ cell population. Their use possibly may help in resolving some inconveniences regarding the validity of results from the antibodies used against the two epitopes.

We are looking forward to hearing from you soon. Please let us know if there is needed more explanation or modification.

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