Author's response to reviews

Title: High infiltration of mast cells predicts worse outcome following resection of colorectal liver metastases

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Author's response to reviews: see over
Dear Dr. Solera:

Thank you for sending us the reviewer’s comments concerning our manuscript entitled “High infiltration of mast cells predicts worse outcome following resection of colorectal liver metastases (MS: 1854567921726039)”. Please find attached the revised manuscript, which has been edited in accordance with the suggestions of the reviewer. The responses to the individual points are detailed below, and the corresponding changes in our manuscript are in red.

We wish to express our sincere appreciation to the reviewer for his/her insightful comments, which we believe have significantly improved our manuscript.

We hope that the revised manuscript is satisfactory and now acceptable for publication in BMC Cancer. Please do not hesitate to contact us if any further revisions are required.

We look forward to hearing from you.

Sincerely yours,

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Reviewer: Jean S Marshall

Comment 1: The authors have used formalin fixed tissue for their study. It is well recognized that formalin is not the optimal fixative for human mast cell visualization as well defined in the older literature. Was microwave antigen retrieval used to enhance the ability to detect mast cells using tryptase immunohistochemistry?

Autoclaving rather than microwaving was performed for antigen retrieval for tryptase immunohistochemistry. In our experience, it is the more reproducible method of the two. Visualization of human mast cells in autoclaved specimens was sufficient for cell counting.

Comment 2: No indication of controls for immunohistochemistry were given, what number of cells stained positively using isotype control antibodies?

We apologize for not including this information. We now state the following: “We used an isotype control antibody to assess antibody specificity (negative control) (monoclonal mouse IgG1 clone #11711; R&D Systems). The control antibody was used in place of and at the same concentration as the primary antibody” (page 8, first sentence in the “immunohistochemistry” section of the Methods). No staining was detected using the isotype control antibody.

Comment 3: More information on the specific cell counting strategy used needs to be provided within the manuscript. Although several papers are referred to for methodology, the strategies used in these references are not identical. How were tumoral and peritumoral areas defined and what area was evaluated for cell numbers (ie mast cell per 10 fields, or for a given number of microns squared).

We defined the peritumoral area as the area that included tumor tissue in 3 of 10 views. Cells were counted in the 3 most abundant peritumoral areas. This information is now provided (page 8, second sentence in the “immunohistochemical evaluation” section of the Methods).

Comments 4: Tryptase staining alone is not appropriate to define mast cell numbers. It is well recognized that tryptase can be taken up by neighboring cells and that macrophages that phagocytose apoptotic mast cells can then stain positively for tryptase. C-Kit staining, FcERI staining or Toluidine blue, would be excellent strategies to confirm findings with tryptase, alternatively pre-staining with Alcian blue at low pH and tryptase immunohistochemistry (as a
double stain) could be useful to confirm that the cells being counted are mast cells.

We agree that double-staining would more specifically identify tryptase-positive mast cells. However, it is very difficult to cleanly stain mast cells for tryptase and a second protein or with a reagent. We did perform toluidine blue staining. The number of tryptase-positive mast cells was slightly less than the number of mast cells stained with toluidine blue. Therefore, we believe that tryptase staining alone is sufficient for specifically identifying mast cells.

Comment 5: Given the existing data that shows a close correlation between microvessel density and mast cells and the known link between highly angiogenic tumors and poor long term disease outcome the authors need to better justify the use of mast cells rather than angiogenic indicators as an approach to predicting survival. Are mast cell counts simply a surrogate marker for vessels?

Mast cell counts are not surrogate markers of vessels. We noted that microvessel density was significantly higher in the high peritumoral group than the low peritumoral group (p < 0.01). However, we were unable to verify this correlation using Pearson’s correlation coefficient, perhaps because the liver is enriched with microvessels.
Reviewer: Girolamo Ranieri

Comment 1: As in background section explained tissue mast cells can be identified via several histochemical methods and immunohistochemical markers as a consequence to make the title manifest I would suggest to specify in it: “mast cells positive to tryptase.”

In accordance with your suggestion, I changed the title to “High infiltration of tryptase-positive mast cells predicts worse outcome following resection of colorectal liver metastases.”

Comment 2: What mean cancer specific survival? Should it to mean Overall Survival? Please explain this. Anyway I would suggest to delete cancer specific survival in that it is not a common definition in international medical oncology community.

Thank you for pointing this out. We used “cancer-specific survival” to indicate overall survival. We have replaced “cancer specific survival” with “overall survival” in the revised manuscript.

Comment 3: In background section, at pag. 5, line 19 the sentence: “Tryptase releases additional angiogenic factors (interleukin 6 and granulocyte-macrophage colony-stimulating factor) from MCs [21], and its degradation aids the invasion and metastasis of tumor cells by induction of angiogenesis [22]” is not clear. How can tryptase to release additional angiogenic factors (interleukin 6 and granulocyte-macrophage colony-stimulating factor) from MCs? In what way or manner? Please specify this. In my opinion reference 21 is not appropriate to explain this. On the other hand reference 21 explain that protease-activated receptor-2 regulates vascular endothelial growth factor expression in breast cancer cells via MAPK pathways.

Thank you for your comments. We have revised our description of tryptase as follows: “Tryptase is an agonist of protease-activated receptor-2 (PAR-2), a transmembrane protein expressed by vascular endothelial cells [21]. Activation of PAR-2 stimulates cell proliferation and the release of interleukin 6 (IL-6) and granulocyte-macrophage colony stimulating factor from MCs, which are angiogenic [22]” (page 5, third sentence in the third paragraph of the Background).

Comment 4: In Patients and Methods sections the employed protocols/schedules of neoadjuvant chemotherapy should be better detailed for group of treated patients. In the cited section the authors reported the following sentence: “Preoperative staging, preoperative chemotherapy, hepatectomy procedures, adjuvant chemotherapy, and patient follow-up were previously described [37,38] ”. From a translational medical oncology point of view the reported sentence is not complete and clear.
In fact in references 37 and 38 a series of 334 and 24 patients has been evaluated respectively while in the present study 135 patients have been evaluated. For example it is important to know how many patients received oxaliplatin that is able to induce hepatic sinusoidal dilatation that in turn could to interfere with mast cells positive to tryptase count. Furthermore I am surprise: are there patients that received bevacizumab or cetuximab?

In our study, 40 patients received oxaliplatin, 17 patients received bevacizumab, and no patients received cetuximab. This information is now provided (page 7, second to the last sentence in the “patients” section in the Methods). We also note the effects of these agents on page 14 (last sentence in the “tumor-infiltrating immune cells and clinicopathological features section” of the Results). We state as follows: “Interestingly, MC counts were similar in patients receiving oxaliplatin and in those receiving bevacizumab.”

I read that the submitted retrospective study included 135 patients who underwent potentially curative resection for CRLM between 2001 and 2010. In Patients and Methods sections, at Immunohistochemical evaluation, line 4 I read: “Micro vessel density was defined as the number of blood vessels, which were identified as areas surrounded by DAB-positive basement membranes”. Is this a standardized method according to Weidner’s or Chalkley’s count? I think no. Please comment this important methodological point for the reproducibility of any experimental data. Please insert specific references.

We have commented on this and have provided a reference in the revised manuscript. We state the following: “Positively stained blood vessels with a lumen as well as cell clusters without a lumen and single cells were considered as individual vessels [41]” (page 8, fifth sentence in the immunohistochemical evaluation section).

Comment 5: In results section, at Patient characteristics, line 5 I read: “The clinicopathological features of patients enrolled in this study are summarized in Table 1”, and in Table 1 no indication regarding RAS status is reported. Why? RAS status should be included in the Univariate and multivariate analyses.

Unfortunately, we have no data on Ras status; it was not determined between January 2001 and December 2010 (the dates of our retrospective study). We know that RAS status influences overall survival in colorectal cancer and will check its status in the future.