Author's response to reviews

Title: VEGFR2 heterogeneity and response to anti-angiogenic low dose metronomic cyclophosphamide treatment

Authors:

Steven G Patten (stpatten@uoguelph.ca)
Una Adamcic (uadamcic@uoguelph.ca)
Kristen Lacombe (klacombe@uoguelph.ca)
Kanwal Minhas (kminhas@uoguelph.ca)
Karolina Skowronski (kskowron@uoguelph.ca)
Brenda L Coomber (bcoomber@uoguelph.ca)

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Author's response to reviews: see over
We thank the reviewers for their thoughtful and helpful comments about this manuscript and we are encouraged by their agreement that our results, while unexpected, are important and should be published. We have addressed the concerns and questions raised in the manuscript; both deleted sections (strikelthrough) and added text are highlighted in yellow. Details of our changes in response to the referee’s specific comments are outlined below. Major changes include a title change and rewording of the manuscript to de-emphasize vessel normalization, additional material in Figure 5 showing VEGF production by SW480 and WM239 cells exposed to 4HC and HIF1a activation, and a new Supplemental Figure demonstrating 5-fold higher levels of VEGFR2 protein in endothelial cells compared to WM239 cells. We trust this manuscript is now acceptable for publication in BMC Cancer, and look forward to a favourable response.

Sincerely

Brenda Coomber

Reviewer #1 – Urban Emmenegger

Major Compulsory Revisions

However, my major concern relates to the proposed role of vascular normalization as a modifier of response to metronomic chemotherapy (pointed to prominently in the title of the manuscript, and discussed throughout the manuscript). The data presented is not convincing regarding the presence of this phenomenon and its potential importance in the context of WM239 and W480 tumor xenografts subjected to metronomic cyclophosphamide chemotherapy.

First, in both tumor models the various parameters analyzed convey contradicting messages about whether vascular normalization indeed takes place.

We agree and have altered the title and re-written the manuscript to de-emphasize vascular normalization

Second, one would expect reduced Hif-1alpha expression levels (indicating reduced tumor hypoxia) in normalized tumors. Could it be that such changes were not seen because whole cell lysates were analyzed instead of nuclear extracts?

This is a possibility, but it is clear from the blots in Fig. 5 that considerable HIF-1a is in fact detectable in several of the samples. Whether nuclear extracts could reveal differences between treated and control tumors, or between colorectal and melanoma tumors is an interesting issue. However, the samples we had in hand were whole tissue lysates, and therefore it is not possible to answer this question at this time.

Third, while vascular normalization is usually considered a
transient phenomenon, Patten et al. only present data of day 15 (WM239) and day 18 (SW480) tumor xenograft specimens, respectively.

**We agree and have altered the title and re-written the manuscript to de-emphasize vascular normalization**

The authors might consider focusing on the aforementioned key findings and de-emphasizing the postulated role of vascular normalization as one way to address this criticism. Key findings such as (i) the lack of predictive information provided by the proportion of VEGFR2+ microvessel density, (ii) treatment-induced reduction of microvessel density despite absent anti-tumor growth effects, and (iii) tumor model dependent differential effects of metronomic cyclophosphamide chemotherapy on a number of ‘vascular’ parameters are worth to be published.

**We agree and have altered the title and de-emphasized vascular normalization and tried to emphasize these points in our revised manuscript**

**Minor Essential Revisions**

Page 2, line 12: should read … VEGFR2 is heterogeneously expressed …

Addressed in the text

Page 9: 4-hydroperoxycyclophosphamide is not a metabolically active form of cyclophosphamide but a precursor of the active cyclophosphamide metabolite 4-hydroxycyclophosphamide.

Addressed in the text

Page 10: should read … the approximate LC50 of 3000 ng/ml is well above the expected tissue levels …

Addressed in the text

Page 12: should read … contained significantly lower proportions of VEGFR2 positive vessels …

Addressed in the text

Page 24: Figure 2 legend should read … treated with 20 mg/kg/day of cyclophosphamide …. Furthermore, the authors may consider to indicate the origin of the tissue section shown in Figure 2D.

Addressed in the text

Page 25: Figure 5 legend should read … and control tumors for either SW480 …

Addressed in the text

Figure 6C: loading control lacking.

**Since we are looking at mural cell recruitment, we have used another vascular marker (CD31), rather than a non-specific loading control such as tubulin for these blots**
Reviewer #2 – Amit Maity

Minor Essential Revisions:

1) There is no change in the %SMA in WM239 xenografts in response to CTX. So there may not be vascular normalization in this particular tumor. Then why is there decreased mean hypoxic area in spite of a decrease in the mean vessel density? This should be addressed in the discussion.

2) On the other hand, there is no change in mean hypoxic area in SW480 tumors. Why is this if there is supposedly normalization in this tumor? This should be addressed in the discussion.

We agree these findings seem paradoxical and have re-written the manuscript to try to account for the apparent contradictions

Reviewer #3 – Darrell Yamashiro

Major compulsory revisions

1. In Figure 1/table 1 there is quantification of VEGFR2 and CD31 staining. From Methods it is unclear how a vessel is defined. The CD31 staining is often discontinuous in a vessel, e.g. Fig 1E, near right *, is this counted as 1 vessel or separate vessels?

Vessel profiles with discontinuous CD31 staining were counted as separate vessels. We did not use an automated imaging program for vessel density calculation; vessels were scored by visual inspection of tissue images in a blinded fashion, thus each structure was evaluated and considered a vessel or not based on its morphological features.

The CD31 staining is also problematic in that autofluorescent RBCs are seen Fig 1E,F on left upper edge of images. Is this corrected by the imaging program?

We did not use an imaging program to score vessels; vessels were scored by visual inspection of captured images in a blinded fashion, thus each structure was evaluated and considered a vessel or not based on its morphological features. DAPI staining for nuclear morphology was used as a reference to determine blood vessel identity; nuclear staining has not been included in images to allow for better visualization of red-green colocalization.

Also, in Methods for xenograft tumors, 5 random fields were picked per tumor, which for SW480 (4 tumors, table 1) is only 20 images to quantify.
We choose 5 random fields to be consistent across every tumor section analyzed in this study. On average, this meant between 50 and 100 blood vessels per tumor section were evaluated.

Lastly, what does VEGFR2 positive staining mean when there is no CD31 staining as there are many VEGFR2+ objects? With these issues it is difficult to interpret their results.

VEGFR2 staining was not counted unless the blood vessel was also positive for CD31, which we assumed for all blood vessels are in our study; other VEGFR2 positive structures could be cancer cells expressing VEGFR2, or recruited bone marrow derived cells.

2. In Figure 4, again the definition of a vessel is pertinent. Fig 4A there is a large “Y” shaped vessel, is this 1 or many vessels?

Branched vessels were counted as a single vessel as long as staining for CD31 and/or VEGFR2 was continuous for the extent of the visible blood vessel profile.

For E-H, the lack of correlation of the Western blots for VEGFR2 with the change in VEGFR2+ vessels is puzzling. They comment that WM239 cells also express VEGFR2 (p13), if true this makes the Western blot uninformative. One could try species specific primers for VEGFR2 and do quantitative PCR.

The addition of Supplemental Figure 1 addresses this issue- although VEGFR2 is expressed by WM239 melanoma cells, endothelial cells express up to 5 fold higher levels and are therefore likely responsible for the majority of VEGFR2 protein detected in these blots.

3. In Figure 5, HIF1a expression is measured. Since VEGF is a downstream target of HIF1a this should be determined. This information on VEGF expression (both between cell lines and with CTX) is critical also for their discussion on VEGFR2 and the potential difference between melanoma and CRC.

VEGF levels in SW480 and WM239 were measured in the presence of CoCl2 treatment (to activate HIF1a); we found in both cases that this ‘hypoxia’ induced additional VEGF production. CTX treatment did not alter VEGF expression, and CTX in combination with CoCl2 was not significantly different from CoCl2 alone. This material is included in a revised Figure 5.

Minor Essential Revisions

4. Figure 6C. The lower gel of CD31 expression in SW480 is too messy to get any meaningful quantification.

We agree that these blots may not be suitable for densitometry and have removed Figure 6D. CD31 blots are notoriously difficult to perform, but we feel that these blots do provide qualitative evidence that desmin levels increase in treated colorectal xenografts, and do not
increase in melanoma xenografts, thus matching what we see with a-sma immunostaining of the same tumors.

Discretionary Revisions

5. In Figure 3A and 3B the MVD is decreased in response to CTX. What marker is used to determine MVD (CD31).

Yes, CD31 was used as our pan-endothelial marker. This has been clarified in the manuscript.

In C & D TSP-1 expression is examined. TSP-1 data would probably be better in a different Figure or in supplemental data, as 3A and 3B is related to Figure 4.

We prefer to leave Figure 3 as is. We think the link between MVD changes and TSP-1 production is relevant and highlights that not all cancer types respond to LDM CTX by similar mechanisms.