Author's response to reviews

Title: Spatial morphological and molecular differences within solid tumors may contribute to the failure of vascular disruptive agent treatments.

Authors:

Linh Nguyen (l.nguyen9@student.unimelb.edu.au)
Theodora Fifis (tfifis@unimelb.edu.au)
Caterina Malcontenti-Wilson (c.malcontenti-wilson@unimelb.edu.au)
Lie Sam Chan (llisachan2012@gmail.com)
Mehrdad Nikfarjam (nikfarjam@unimelb.edu.au)
Vijayaragavan Muralidharan (v.muralidharan@unimelb.edu.au)
Christopher Christophi (cchri@unimelbedu.au)

Version: 3 Date: 17 August 2012

Author's response to reviews: see over
Dear Sir/Madam,

Please find attached a revised version of the manuscript entitled: “Spatial morphological and molecular differences within solid tumors may contribute to the failure of vascular disruptive agent treatments.” by Linh Nguyen, Theodora, Caterina Malcontenti-Wilson, Lie Sam Chan, Patricia Luiza Nunes Costa, Mehrdad Nikfarjam, Vijayaragavan Muralidharan, Christopher Christophi for consideration for publication in the Journal of BMC Cancer in the original manuscript section.

We thank the reviewers for their suggestions and constructive criticisms. We took these suggestions/criticisms into account in this revised manuscript.

With best Regards

Theodora Fifis

Addressing reviewers' specific points

Referee 1:

Major Compulsory Revisions

1) On pages 6 & 7, the authors state the numbers of tumours used in the analysis but information should be given about numbers and sizes of tumours in each liver and whether the whole of the liver tissue was used. Statistical analysis needs to take into account inter-animal variation as well as inter-tumour variation and error bars should reflect this. It is not clear whether this has been done and statistical advice should be sought.

We have published this information on this model before (Chan et al 2007), however we now include a supplementary figure illustrating how tumors are processed and analysed. In this model tumours of various sizes are spread throughout the liver. If metastases are seeded in close proximity they coalesce into a continuous tumour. Like in other tumour models VDA has a greater effect on large vascularised metastases. The metastasis pattern is very similar and reproducible within a group of mice.

2) The methods for quantifying the staining data are unclear and need to be described in more detail. It is unclear whether counts were made by a fully automated method or whether there was manual input. In either case, the criteria for assessing ‘positively stained vessels’, for instance, should be given. Similar details need to be given for CD3, FOXP3 and F4/80. Quality control methods and whether analysts were blinded need to be stated. The axis labeling needs some clarification. For instance, in Fig 1, CD34 positive MVD is not very informative and should be replaced by ‘number of vessels per mm^2’, if this is what the graph shows. Similarly, ‘pericyte coverage’ needs defining clearly.

All these points have now been addressed.

3) Quantified results should be provided for hypoxia, VEGF, FGF, HIF-1alpha, AT1R, TGFbeta.
We present quantified results for VEGF, AT1R and TGF-b. Hypoxia and HIF-1alpha although both show reduced levels or absence in the periphery they display considerable heterogeneity in the bulk of the tumor depending on the distance from major vessels, Therefore quantification is neither easy or meaningful. Unfortunately b-FGF staining was performed a while ago and the sections have since been damaged and cannot be accurately quantified. We however chosen a different section representing a clearer staining to support our claims

4) Results – Fig 1.
The antibody for CD34 is clearly only staining a small fraction of vessels, which appears to be the larger ones. The authors’ justification for using CD34 as their endothelial cell marker is that it is expressed on vessels undergoing neoangiogenesis and cite unpublished work to support this statement. However, there is no direct evidence for this.

CD34 is one of the markers used for tumor vessel staining in other publications. Like most antibodies for vessel staining, it is not a perfect marker however in our experience it stains tumor vessels but not liver vessels unless the liver is regenerating after hepatectomy. CD31 is another antibody used for vessel staining, however some reports in the literature claim it to be more cross-reactive than CD34. Taking the reviewers suggestions we have now used CD31 staining and we found it stains roughly equal numbers of tumour vessels but it also displays strong staining of liver sinusoids and vessels. (Supplementary Figure)

The images for #-SMA staining for pericytes are rather poor quality. Better images are required to demonstrate that there is more #-SMA staining on vessels in the periphery than in the centre. Some lower power images should be shown, as has been done for CD34.

The particular tumor we using stimulates high myofibroblast activity and strong a-SMA staining therefore a lower magnification totally masks the vessel staining. However we now provide a lower magnification of a-SMA staining and included this information as Supplementary Figure.

CD31 staining should also be carried out, as this should highlight many more vessels and aid in identifying #-SMA co-localised to vessels. It is not at all clear how the pericyte coverage data was calculated, considering the high level of #-SMA staining shown in C in non-vascular structures, presumably myofibroblasts. Authors need to explain how this was done and define ‘pericyte coverage’ (see above).

As stated above CD31 does not stain more tumor vessels than CD34. Due to high a-SMA staining of myofibroblasts in this model double staining with CD31 and a-SMA does not improve the resolution of pericyte containing vessels. We now include more details of how pericyte coverage was assessed.

5) The Ang1 data is insufficient to back-up the conclusion that there are more mature vessels in the periphery. Evidence from #-SMA staining should be strengthened, as above, or a second pericyte marker should be included (see below).
We present a different angiopoetin 1 stained section (Supplementary Figure) that more convincingly demonstrates preferential peripheral vessel staining.

6) Fig 3
Results for these markers should be quantified to support the statements made in Results. In particular, distribution of HIF1alpha did not obviously mirror pimonidazole staining, as stated in the text. These do not appear to be serial sections and so it is difficult to see the evidence for this statement. Although there is very little pimonidazole staining at the periphery of the tumour shown, the HIF1alpha staining appears to extend to the periphery.

It is true HIF1a is also seen in areas that there is no hypoxia staining, however in hypoxic areas HIF1a is more concentrated. This has been addressed in the revised manuscript.

B-FGF staining is unclear – does the expanded area include normal liver as well as tumour tissue? There is a lot of positive staining for b-FGF and TGF-beta in normal liver – how do controls compare with these? Is this specific staining? These issues need to be clarified.

Normal liver shows some staining with both b-FGF and TGF-β, however this staining increases in livers bearing metastases especially adjacent to tumors. We have shown in a previous publication (Fifis et al 2011) that TGF-β increases significantly in the liver parenchyma of mice with CRC metastases, furthermore liver expresses significantly more TGF-β compared to tumor tissue.

7) Fig 6
Extensive necrosis is seen at 1h after treatment in the figure. Is this typical across the tumours? The necrosis data should be quantified for all the tumours over the full time-course to show overall response to treatment and a comment should be made on how this compares with other tumour models in the literature.

This was the subject of an earlier publication. In this model extensive necrosis is observed which is more pronounced in the larger tumours. This is similar to that reported in many other tumor models although there are certain tumors that demonstrate more resistance as we already discussed the response to VDA treatments in other tumour models in this manuscript.

Minor Essential Revisions
1) Fig 1
The arrows in A appear to be indicating vessels outside the tumour mass. Since the paper is concerned with vessels in the tumour periphery, this should be changed.

These vessels are indicated as host vessels in immediate proximity to tumor periphery that are being co-opted by tumor.

2) Fig 5
What does the asterix in Fig5C (24h) signify?
That is an error it should be a double asterix indicating significant reduction in the number of in CD34 staining vessels at 24 hrs after treatment in the tumor center. That has now been corrected.

What evidence is there for tumour vessels being leaky at 1h after treatment? It is not possible to tell this from the figure and the statement should be deleted.

This has been changed accordingly.

The statement in the text about patent vessels in the periphery at 24h should refer to the 24h image, not the 1h image, I think.

This has been corrected.

3) Discussion
Repetition on page 14, in paragraph 2.

This has been corrected.

Discretionary Revisions
1) A second pericyte marker would help to clarify the issue of whether the vessels at the periphery are more mature than in the centre, since the Ang1 result is unconvincing. NG2 would be a possibility.

NG2 is another marker used for vessel maturity, however it does not recognise all vessels and it shows significant cross reaction with other cell types. and It takes time to optimize antibody staining and with the possibility of no additional information gained we think that NG2 staining is beyond the scope of this study. We demonstrated that vessels in the periphery are more resistant to treatment by demonstrating patency following treatment. Association with pericytes and Ang1 provide some explanation for this resistance but does not change the finding.

2) It would be helpful, in Methods, to group the antigens into different categories e.g. FOXP3 and CD3 are for T-cells etc.

This has been done.

3) Were any other markers investigated that did not have higher expression at the periphery? – this would be useful information too.

We have preliminary evidence of other markers such as HGF that are preferentially expressed in the periphery but at this stage no other markers that are expressed less.

Referee 3:
Reviewer's report:
In this manuscript Nguyen et al have investigated morphological and molecular characteristics of the tumour, its vasculature and stroma using a colorectal liver metastasis model and an immunohistochemical approach. The authors suggest that inherent differences between the periphery and central regions of the tumour could contribute to the failure of vascular disrupting agents to effectively target the tumour rim. The data presented in this study are very interesting but mainly correlative – there is no direct evidence provided here that the morphological and molecular differences described are directly responsible for the failure of VDAs. I would therefore suggest that the title of the manuscript is changed.

The title has been modified.

Major points

1. In page 8 of the manuscript the authors state that vessels in the tumour periphery either did not stain or only partially stained for CD34. Presumably they refer to the rather large open vessels that could be easily distinguished morphologically. However, tumour vessel morphology/size is heterogeneous and this raises the question of whether the data presented on microvascular density throughout this manuscript reflects the true extent of vascularisation of these tumours. For example, from the images presented it is not clear whether the antibody picked up smaller vessels at all? The authors should consider testing another endothelial marker such as CD31 in their system.

In the literature CD34 or CD31 are used to quantify tumor vessel density. Controversy exists as to which of the two is the most appropriate and whether one or the other stains the immature vessels. Reports in the literature claim that CD31 in addition to endothelial cells also stain platelets and sinusoids. For this reason we decided to use CD34. In this study we found it preferentially stains central tumor vessels while minimally stains host vessels or sinusoids except in the regenerating liver (unpublished result). We took the reviewer’s suggestion and also stained with CD31. The results show that both antibodies stain roughly equal number of tumor vessels while CD31 also strongly stains sinusoids and host vessels. These results are presented as a supplementary Figure.

Also the authors should describe how they quantified MVD and pericyte coverage in more detail and explain what the numbers in the bar charts presented in Figure 1 mean?

MDV was quantified by counting the number of CD34 staining vessels per mm². This has now been clarified in the figure and in the methods. The second graph represents vessels demonstrating some pericyte coverage as detected by a-SMA staining.

Also, how were pericytes distinguished from myofibroblasts if not all blood vessels could be detected.

As the reviewer noted there is extensive staining with a-SMA presumably of myofibroblasts in this tumor and this makes it challenging to distinguish all vessels with pericyte coverage based on a-SMA staining. We found this particularly difficult using double staining. For this reason we carried out single staining for each marker on serial sections and focused on vessels that stain with a-SMA. Pericytes are cover the vessels and appear like thin lines
along the vessels (indicated by arrows in supplementary figure 3). In contrast myofibroblasts show strong cytoplasmic staining throughout the tumor bed and more so in the periphery.

2. Figure 3: the central region of the tumor in the top left panel looks quite normoxic? In addition, there are rim areas within the same section (to the right) that look quite hypoxic? The authors should provide quantification of the hypoxic and normoxic tumor area in relation to spatial distribution.

In our results we stated that hypoxia is variable throughout the tumor depending how distant the tumors are from a major vessel, so areas within the tumor close to major vessels are normoxic. The reviewer is right there are areas in the tumor periphery that hypoxia is evident, however these are areas where the tumor is growing on the liver surface with no adjacent liver parenchyma and host vessels. We now show a different section showing these two cases clearly and we described them in more detail in the results. We do not believe quantifying the hypoxia regions will be meaningful or easy to do due to the observed heterogeneity within the tumor center and depends on the size and vascularization of each tumor, however our qualitative staining clearly demonstrates that the tumor periphery is by e{n} large normoxic.

3. Figure 3: how representative is the VEGF peripheral staining shown in the middle row of this Figure? Did all tumours stain similarly?

Figure 3 is a representative of several different tumor sections from a minimum of five mice. Each liver contains several tumors of varying sizes including coalescing metastases. When the tumors are very small, VEGF staining extends almost throughout the tumor while larger tumors stain mainly in the periphery and in a number of tumors staining is also seen in major vascular lakes within the tumor center. Interestingly when such staining occurs within the tumor these areas appear more resistant to treatment at 1hr however by 24 hours generally it is only in the periphery that tumor survives. As reported in the manuscript staining closely mirrors macrophage distribution.

4. Figure 3, bFGF staining: where does the tumour tissue end and liver parenchyma begin? Parallel H&E stained sections would be useful here.

As stated in manuscript the liver parenchyma of tumor bearing mice strongly stains with b-FGF in areas close to the tumor. We do not have the original paraffin block for H&E staining as suggested by the reviewer, instead we present another b-FGF stained section that clearly shows tumor/host interface.

5. Figures 5 and 6 caspase staining: if vessels in the central regions failed to stain with CD34, then how did the authors localize and quantify caspase activity in the vasculature of treated tumours? How were caspase-positive tumour vessels defined and how was the distinction made between vascular endothelial and tumour cell apoptosis?
Vessels in the central region preferentially stain with CD34. At 24 hours after treatment the majority of these vessels were destroyed and no longer stain. We only counted vessels that double stained with CD34 and caspase. Caspase staining was seen both in endothelial cells lining the tumor vessels and in adjacent tumor cells, however for the calculations only CD34 positive vessels that also stained positive with caspase in double staining as shown in supplementary figure 4 were counted.

What was the level of apoptosis in control untreated tumours?

We assume the reviewer is asking whether we seen endothelial cell apoptosis in the vessels of control tumors. There was negligible apoptosis. We present a new supplementary figure showing the double staining in control and treated tumors. The control does not display any double staining while this is strongly evident in the treated tumors.

6. Page 11. The authors state that the vessels became “leaky” after Oxi4503 but have not assessed this with any functional assays.

The reviewer is correct we did not present evidence of vessel leakiness in this study. We have published this finding in a previous publication. We have changed the text to address this issue.

Minor points
7. The authors state how many tumours they analysed per treatment group but do not say how many animals per group these represent. This information is needed for each of the analyses presented.

Number of animals per group is now stated in materials and methods section and in the relevant figures.

8. All figures need appropriate scale bars
This has now been addressed.