Reviewer’s report

Title: Transcriptional profiling elucidates metronomic cyclophosphamide-activated, innate immune-dependent regression of brain tumor xenografts

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Reviewer: Eddy Pasquier

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The manuscript entitled “Transcriptional profiling elucidates metronomic cyclophosphamide-activated, innate immune-dependent regression of brain tumor xenografts” by Doloff and Waxman presents the results of mouse and human microarrays performed on brain tumor xenografts treated with metronomic cyclophosphamide. This is a very interesting study, which is also very timely. Indeed, metronomic chemotherapy is gaining considerable interest in the clinic, having demonstrated its efficacy in a variety of human tumours. In-depth mechanistic studies are now warranted to unravel the molecular factors involved in its anti-tumour effects and ultimately identify patients that are most likely to respond to this type of treatment.

This study has two major weaknesses. The first one is the use of heterotopic implantation of tumours. Indeed, the focus of the study is on the tumour micro-environment and especially anti-tumour immune response. Therefore, orthotopic (i.e intracranial) implantation of tumour cells would have been much more appropriate to investigate the impact of treatment on local immune response. Having said that, the authors have previously published several articles using this sub-cutaneous model of glioma and have characterized its response to metronomic cyclophosphamide. This allowed them to correlate the changes in gene expression observed in the present study, with tumour regression and immune system activation previously reported in the same model.

The second weakness of the study is that it is purely descriptive and crucially lacks functional validation. The authors describe at great length the 1,447 human and 614 mouse genes whose expression was significantly altered as a result of metronomic cyclophosphamide treatment, alongside their associated pathways and upstream regulators identified by bioinformatics analysis. However, none of these changes in gene expression and activation/inhibition of upstream regulators are confirmed at the protein level (except for some rare, retrospective validation like for the NKG2D activating ligand MICB). Furthermore, none of these changes have been functionally validated either by RNA interference or using pharmacological inhibitors/activators. Key molecular events such as activation of the interferon pathway, increase in CXCL9-11 cytokines or inhibition of MAPK1 and ERK1/2 could be quite easily validated by ELISA or western blotting. This would considerably strengthen this study.
Despite these obvious limitations, this study provides numerous insights into the mechanism of anti-tumour immune response induced by metronomic cyclophosphamide.

MAJOR COMPULSORY REVISIONS

1) The title of the article should be revised to better reflect the content of the manuscript. Indeed, descriptive studies do not “elucidate” a mechanism. Elucidation requires more than correlative studies: it needs functional validation. I suggest using the wording “provides insights” instead of “elucidates”.

2) The structure of the discussion is somewhat tortuous and sometimes sounds like a catalogue of genes involved in anti-tumour immune response. The authors should work on their discussion to make it flow better and to avoid repetitions (e.g results obtained on MICB are briefly discussed page 16 and then again at great length page 19).

3) It is pretty obvious that among the changes in gene expression reported in this study some will be beneficial to the anti-tumour effects of metronomic cyclophosphamide while others will be detrimental. Some might be direct effects, while others might be part of compensatory mechanisms. Although the authors presented a small selection of deleterious changes in Table 1, they failed to discuss any of those, although they did mention these detrimental changes in their overall conclusion to underline their importance. These changes and their potential therapeutic implications should be included in the discussion. For instance, has MMP13 up-regulation been previously implicated in chemo-resistance? Could it be targeted using pharmacological inhibitors and/or therapeutic antibodies to improve the efficacy of metronomic chemotherapy? Similarly for CEACAM1, IDO1…

4) Similar deleterious changes have been observed in the mouse array (e.g activation of SAMSN1, inhibition of CSF2…) and should be included in the discussion.

MINOR ESSENTIAL REVISIONS

5) Page 3 – the sentence “several cytokines and chemokines […] were also identified…” suggests that references #16 and #17 investigated the mechanism of action of metronomic chemotherapy, which is not the case. This should be rephrased or simply removed as it is confusing and the introduction would flow much better without.

6) Page 4 – the sentences “Tumor regression is not, however, a secondary response […] rather it is a direct consequence of the mobilization of innate immune cells…” How was this demonstrated in references #20-23 ? This should be stated.

7) Page 5 – How was the cyclophosphamide administered? Orally, by i.p, i.v injection? This should be clearly stated.
8) Page 6 – in the sentence “Fluorescent labeling of RNA […] in expression of host cell (mouse) RNAs” a verb is missing. This should be rephrased.

9) Page 7 – The authors suggest that the low number of genes showing opposite regulation at the two time points (8) underlines the reliability of their approach. But does it really? If more genes showed such opposite regulation at the two time points, it would not necessarily mean that the microarray analysis was not significant. It could also mean that changes in gene expression are more transient and/or time-dependent. This sentence should be rephrased.

10) Out of the 1,447 genes whose expression was either up- (806) or down-regulated (641) at both treatment time points, only 19 are presented in Table 1. This suggests that only 19 of these genes could be classified as either beneficial or undesirable, which is not the case, I assume. The authors should explain the criteria that were used for this selection (e.g. lowest p-value? Highest fold change?).

11) The readers might not all be familiar with the IPA software. Therefore, the meaning of the different shapes (e.g. circle, oval, diamond, square…) and lines (solid, broken, coloured…) should be explained in the figure legends (Figures 1 & 3).

12) Page 13 – the sentence “the up-regulated gene cluster showing the second highest enrichment…” should be moved up to directly follow the sentence ending by “Table 4A”.

13) Page 20 - there is a typo in the second sentence: "distinguishing distinguish self"

14) Page 21 – ref #103 does not really support the statement that “CPA and other drugs that activate immunogenic cell death are increasingly being studied in combination with immunotherapies”. We and others have recently reviewed the combination of immunotherapy with metronomic chemotherapy (Andre et al., Nat Rev Clin Oncol 2014; Sheng Sow et al., Oncoimmunology; Nars et al., Int J Cancer 2013). Any of these references would be more appropriate to support this statement.

DISCRETIONARY REVISIONS

15) The authors evaluated the microarray probe specificity for the human and mouse genome by performing BLAT analysis. It could be interesting to undertake the same analysis with the rat genome to determine the overlap between the mouse array and the rat genome.

16) Page 19 – The authors mentioned that they have found that “CPA-damaged gliomas secrete HMGB1 protein” but this refers to unpublished experiments. Why was this not included in the present study? This is the kind of validation that would greatly enhance the significance of this work.
Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interests