Reviewer's report

Title: VEGFR2 heterogeneity and response to anti-angiogenic low dose metronomic cyclophosphamide treatment: role of vascular normalization

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Reviewer: Urban Emmenegger

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The lack of predictive markers is among the major shortcomings of antiangiogenic tumor therapies. Therefore, by focusing on vascular heterogeneity (i.e., proportion of VEGFR2+ tumor microvessels) as one potential explanation of differential response to metronomic cyclophosphamide therapy, the manuscript by Patten et al. is very timely and innovative. Having shown earlier that variable expression of the Tie2 receptor tyrosine kinase in certain xenograft tumor models affects response to Tie2 inhibition, the authors hypothesize that the proportion of VEGFR2+ microvessels may impact on the effectiveness of metronomic chemotherapy.

Using colorectal and melanoma human tumor xenografts, as well as colorectal cancer and melanoma human tissue arrays, Patten et al. document different percentages of VEGFR2+ microvessels in colorectal cancer (~60%) versus melanomas (~90%). Interestingly, the proportion of VEGFR2+ microvessels in melanoma specimens compared to WM239 melanoma xenografts, and in colorectal cancer samples compared to SW480 colorectal cancer xenografts, respectively, are very similar. Despite any significant impact on tumor growth rates, metronomic cyclophosphamide therapy reduces CD31+ and VEGFR2+ microvessel density in both xenograft tumor models. Thus, the proportion of VEGFR2+ microvessels appears not to predict response to metronomic cyclophosphamide chemotherapy if reduction in microvessel density is used as a read-out. Otherwise, metronomic cyclophosphamide therapy affects parameters such as CAIX, TSP-1, and desmin expression, as well as alpha-SMA+ microvessel density differently in the two tumor models analyzed.

The experiments are well executed, and the manuscript is clearly written. A few minor criticisms of the data presentation are summarized below. 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. Having said this, in its present form the manuscript has also certain shortcomings. Unfortunately, Patten et al. show only data from two treatment-refractory tumor models. The findings of the same type of analyses in a metronomic cyclophosphamide sensitive tumor model could provide more comprehensive information. Furthermore, it is currently not known whether the VEGF/VEGFR2 axis is indeed a major target of metronomic
chemotherapy. Therefore, as the authors state, it will be important to investigate whether heterogeneity in the proportion of VEGFR2+ microvessels may account for differential responses to targeted anti-VEGFR2 therapy.

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. 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? 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. 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.

Minor Essential Revisions

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

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

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

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

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.

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

Figure 6C: loading control lacking.

Level of interest: An article of importance in its field

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.