Author’s response to reviews

Title: Evaluation of the impact of transient interruption of antiangiogenic treatment using ultrasound-based techniques in a murine model of hepatocellular carcinoma

Authors:

Sara Marinelli (sara_marinelli@libero.it)
Veronica Salvatore (veronica.salvatore@unibo.it)
Marco Baron Toaldo (marco.barontoaldo@unibo.it)
Maddalena Milazzo (maddamilazzo@hotmail.it)
Luca Croci (luca.croci5822@gmail.com)
Laura Venerandi (laura.venerandi@gmail.com)
Anna Pecorelli (pecorelli.anna@gmail.com)
Chiara Palamà (chiarapal@live.it)
Alessia Diana (alessia.diana@unibo.it)
Luigi Bolondi (luigi.bolondi@unibo.it)
Fabio Piscaglia (fabio.piscaglia@unibo.it)

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Author’s response to reviews:

Dear Editor,

thanks for giving us the possibility to submit again our paper to your journal, now improved thanks to reviewers’ suggestions. Please find below a point by point reply.

Reviewer 1:

Nice manuscript that highlights 2 points; First confirms "rebound" effect of neovasculature following withdrawal of Sorafenib, and Second – demonstration of CEUS technology in preclinical model of cancer.

Minor comments:

1. Authors should add (if possible) matching histology /histochemistry slide showing the rebound of tumor after cessation of treatment to strengthen their claim.

  Re: We thank the reviewer for his precious suggestion. We performed also the histopathology analysis of all masses that confirmed our hypothesis by showing more vessels in group three compared to the others. Paragraphs have been added (in M&M section and in the results section) as well as an image that compares the three groups.

2. Avoid some redundancies in introduction/discussion section.
Reviewer 2:

In this study, Marinelli et al evaluated the impact of interruption of sorafenib (an antiangiogenic drug) treatment in a murine model of HCC. The volume, elasticity and VEGFR2 of the tumors were measured by B-mode, elastosonography and molecular-CEUS ultrasound examinations at different time points. Finally the authors detected the levels of VEGFR2 in tumors by using western blot analysis.

Overall, the evidence presented appears to support the authors' main conclusion: a neoangiogenetic rebound after sorafenib treatment withdrawal in a murine model of HCC. However, several specific concerns are described below:

1. The amount of mice in this study is limited, and each group has different amount of mice. If more mice could be randomized in different groups equally, the data would be more convincing.

Re: We know that the amount of mice is limited but it is line with other studies using animal models (e.g. Kotopoulis S, Mol Imaging Biol, 2014; Fox WD, Clin Cancer Res, 2002; Nagengast WB, Cancer Res, 2010 and many others), which often include less than 10 animals each group. Twenty mice have been included originally in the study but tumors grew only in 16 of them, as often happens in animal models. The animals were then randomized in three groups. During the 13-days protocol, 2 animals (1 in placebo group and 1 in sorafenib-placebo group) with large tumor masses were found died in the cage and for this reasons they were excluded from the analysis. This may support our hypothesis of progressive tumor increase after sorafenib withdrawal and it has been included in the manuscript.

2. A sorafenib treated mice group without interruption should be added as control.

A mice group treated continuously with sorafenib was not initially set up since the dose- and time-dependent inhibition of VEGFR2 has been already demonstrated (Mei J, Cell Biochem Biophys, 2013) and it is a well-known important target and for this reason. Our group have been already studied VEGFR2 expression in the same tumor model in a previous experiment (actually under review) but it would be incorrect to compare now the quantification of different experiments since the same reference standard for quantification cannot be utilized. Therefore, to this end not just one additional group but the entire experiment should be repeated from the beginning which implies excessive time, costs and a new submission to the Ethical Committee which might not found justified so many new animals sacrificed to have little additional information.

3. The data of figures 2 and 4 were measured at different time points, if they could be presented by histogram with standard deviation instead of curve diagram it would be more straightforward.
Re. Figure 4 (now figure 6) has been changed following your suggestion. Nevertheless, we prefer to not change figure 2 because it refers to dimensional changes at three time points with respect to day 5 and our impression was that the corresponding histogram would be more confusing.

4. The data of VEGFR2 western blot analysis and percentage of non-enhanced areas in table 1 should be shown in detail instead of median or average value.

Re. Table 4 refers to percentage of non-enhanced areas without mentioning VEGFR2 Western-Blot results. Following the suggestion we reported in details percentage of non enhanced areas of each animal. However, reporting quantification of each WB would be quite boring for readers and poorly impacting. However, we understood the request of the reviewer for the benefit of the readers and we thought it was more interesting and illustrative to show directly representative images of the Western-Blot findings (figure 4).