Author's response to reviews

Title: Irresponsiveness of two retinoblastoma cases to conservative therapy correlates with up-regulation of the VEGF-A pathway.

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Author's response to reviews: see over
Dear Editor,

We submit a revised version of our manuscript former entitled “Irresponsiveness of two retinoblastoma cases to conservative therapy correlates with up- regulation of the VEGF-A pathway”. We have changed the title of the manuscript which is now “Irresponsiveness of two retinoblastoma cases to conservative therapy correlates with up- regulation of hERG1 channels and of the VEGF-A pathway” to fulfill Reviewer #1’s suggestion. In fact, we have studied in more detail the expression of hERG1 channels and immunohistochemical staining of the two retinoblastoma (RB) cases has been reported in the new Figure 3. Hence the term “upregulation of hERG1 channels” has been included in the title of the manuscript.

The text has been revised and more details on the follow up course of the two cases have been added to address Reviewer #2’s criticism.

Figure 3 has been fully redrawn: panel C now shows Hematoxylin and Eosin (H&E), as well as immunohistochemistry of VEGF-A and hERG1 of the two RB cases; panel D shows a Table with data relative to the percentage of stained cells (for VEGF-A and hERG1), microvessel density (MVD), proliferative and apoptotic index to address Reviewer #1’s criticisms. Changes in the text are highlighted in red/bold.

Our point-by-point response to the reviewers’ comments is as follows:

Reviewer #1:

Major points:

1. Genetic analysis of retinoblastoma gene (RB1) was performed on DNA extracted from peripheral blood lymphocytes. We specified this in the text (page 7 and 8).
2. The term aggressive has been removed.
3. New references on angiogenesis have been added in the revised manuscript (references 14, 15, 16, 17).
4. The proliferative and apoptotic indices (PI and AI, respectively) of both cases were assessed by IHC (Ki-67 antigen and FragEL staining, respectively). The two parameters are reported in the Table of panel D of the new Figure 3.
5. Figure 3 C has been fully redrawn and the new Figure 3 shows histology (H&E) and immunohistochemistry (for VEGF-A and hERG1) of
both RB cases. Larger fields and two magnifications (200x and 400x) are shown, for each picture.

7. The microvessel density (MVD) has been evaluated on both RB cases and results are shown in the Table of panel D of the new Figure 3.

8. The number and intensity of stained cells for VEGF-A have been evaluated as described in Arean et al, 2010 and are now reported in the Results and in panel D of the new Figure 3. The same procedure has been applied to quantify hERG1 staining.

MINOR essential Revisions
1. The reference numbering appearance has been checked.
2. The manuscript has been revised for slips.
3. We have measured more carefully the dimension of the tumour mass of case 1; it resulted to be 9.77 mm height and 12 mm at the base. These new data are indicated in the text (page 8).
4. The echographic profile of the tumour mass in Figure 1A is now indicated with white arrows.
5. We have detailed the follow up of case 1 and 2 (see also the answers to reviewer #2) and details are reported in the text (page 8 and 9). Case 2 responded to chemotherapy with a 50% reduction of the tumour size. Such response was maintained for 2 years when a sudden recurrence at a different site was observed.

Reviewer #2:
Major points:

Major Compulsory Revisions
1. We chose the term “irresponsiveness to conservative treatment” to address the two RB cases described in the manuscript, since both cases turned out to behave differently from all the RB cases observed and treated with conservative therapies in the Department of Pediatric Ophthalmology Meyer Hospital of Florence, in the same period. In fact, both cases experienced an initial response, although partial, to chemotherapy. Such only partial response was maintained during the follow up period, which lasted 5 years for case 1, and 2 years for case 2.
At those times a sudden recurrence was observed, in sites different from those presenting the lesion at the diagnosis. We have now described in more detail the disease course during the follow up period for both cases (page 7-9).

2. We compared the biomolecular data (vegf-a, flt-1, kdr, gapdh, hif-α, herg1a and herg1b mRNA by RQ-PCR) between the cancerous and the normal tissue from the same patient. On the other hand, VEGF-A secretion data refer only to the vitreous of the two patients. We compared such values with that reported by Sonmez et al 2008 (references n° 22 of the new manuscript) for age-paired patients (see dotted line in figure 3B). This is now better clarified both in the text and in the legend to Figure 3 of the revised manuscript. We couldn’t determine the VEGF-A secretion in the vitreous of patients affected by RB responsive to conservative therapy, since the Ethical committee of the Pediatric Meyer Hospital does not authorize vitrectomy of an eye with RB, to avoid dissemination of the tumour and hence to prevent metastasis (see Zografos Leonidas “Tumeurs intraoculaires” Edited by Masson 2002 p. 612).