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Response to reviewers

## Reviewer 1:

The manuscript presents results of transcripts and metabolic pathways analyses in retinoblastoma tumors compared with the normal retina. The assays were performed in tumor biopsies, for RNA transcripts characterization, and in the vitreous humor, for metabolites analysis, obtained from retinoblastoma patients. The microarray method identifies differential expression of genes, encoding for several biological pathways, between clinically advanced and non-advanced tumors. These results were validated by quantitated RT-PCRs. The metabolomic analysis identifies some enriched metabolic pathways in the surrounding tumor media. Metabolic pathways of the humor were compared with the transcripts presented in tumor for the correlation of both and thus, for obtaining an integrated analysis. The analysis of gene expression was also performed at protein level by immunohistochemistry, studying several characteristic proteins like Ki67, E2F2 and pRB. This study was completed with the characterization of those protein in retinoblastoma cell lines.

This work was well performed and is very interesting, I have some commentaries for better understanding of the results:

Author's response: We thank the reviewer for the critical review and comments which have improved the manuscript overall. We address each of the concerns raised point-wise below.

Reviewer 1 comments:

1) Explanation of the meaning of gene acronyms, such as those mentioned in the text (Lines 435-440 and Fig. 5B): HDAC, FN1, BAAT, HIF1A, PRKAA2 (AMPKalfa), GUCA1A and their function in metabolic pathways.

   Author's response: As pointed out by the reviewer, we have expanded the gene acronyms and have added them to the text. We have also addressed the reviewer's comment regarding expansion of the explanation of the observations with regards to functions of the selected genes in metabolic pathways and relevant references are cited in the text (page15, Results section, lines 435-452).

2) From which patient the normal retina was obtained?

   Author's response: The two pediatric retinae used in the study were obtained from 3month old and 2month old children who died of non-ocular complications. The detailed information of the samples is provided on page 3, Methods section, Table 1, lines 115-117.

3) In Figure 5A are marked the predominant metabolisms: fatty acids, amino acids and pentose phosphate. Compared with Figure 4D heatmap all the results (Fig 4D, Fig. 5A/B) agree for fatty acids as is stated in the text (line 426/427). However, not all amino acids predominate in humor (Fig 4E and line 426/427).

   Author's response: We agree with the reviewer's findings that not all amino acids are predominantly elevated or reduced in Rb vitreous humor. Our data show that a subset of the amino

acids namely creatine, taurine, and isoleucine-histidine-lysine displayed varied regulation in Rb subtypes (mentioned on page13, Results section, lines 390-392) while glycine, serine, and methionine show poor enrichment. Hence, we agree with the reviewer's observation and have revised the sentence in the text (page 15, Results section, line 427).

4) Line 434 and Figure 5B: Valproic acid is a drug used in brain diseases and it is derived from naturally occurring valeric acid. How can the authors explain its presence in human's tissues?

Author's response: We agree with the reviewer's observation on valproic acid is a derivative of valeric acid and used as a therapeutic. Structurally similar valproic acid-like metabolites were identified in berries, chocolates, and tea as mood-enhancing chemicals in the human diet *(Martinez et al., Good mood foods: Some flavors in some foods resemble a prescription mood stabilizer." American Chemical Society (ACS). August 2012).* Valproic acid is a branched-chain fatty acid derived from valeric acids, which is present in human diet and processed by the gut bacteria (*Tan J et al.,The role of short-chain fatty acids in health and disease. Adv Immunol. 2014).* Its ability to transport from gut epithelium to the bloodstream and into ocular tissues was reported in animal models (*Skrzypecki J et sl., Valeric Acid, a Gut Microbiota Product, Penetrates to the Eye and Lowers Intraocular Pressure in Rats. Nutrients. 2020).* Our study showcases multiple valeric acid derivatives in Rb vitreous humor, highlighting their presence in the human diet and their penetrating ability in ocular tissues, of which valproic acid was identified as a top downregulated metabolite. Of note, we identify Valproic acid as reduced and so is Valeric acid (Pentanoic acid) which probably has biological significance in terms of the uptake and processing pathways being altered in the tumors.

5) Taurine is downregulated (line 436 and Fig. 5B), however taurine metabolism is enriched (Fig. 4E). For example: BAAT gene (bile and CoA amino acid [glycine, taurine] N-acyl transferase) is highly expressed (line436 and Fig. 5B) but taurine is in low abundance (line 437 and Fig. 5B). How can be explained these results?

Author's response: The highly expressed BAAT gene is known to catalyze the bile acid conjugation by utilizing either glycine or taurine, affecting bile acid synthesis and mitochondrial metabolism (*Kirilenko BM et al., Evolutionary Analysis of Bile Acid-Conjugating Enzymes Reveals a Complex Duplication and Reciprocal Loss History. Genome Biol Evol. 2019).* Therefore, there is a greater amount of flux in the taurine metabolism and since both glycine and taurine are reduced in our study, it indicates their utilization for subsequent biosynthetic pathways in Rb. Hence, the high BAAT expression likely leads to a rapid utilization of Taurine and Glycine. Incidentally, Taurine deficiency is reported in glioblastoma cancer and the enriched taurine/hypo-taurine metabolism drives glioblastoma progression through hypo-taurine accumulation *(Shen D et al., ADO/hypotaurine: a novel metabolic pathway contributing to glioblastoma development. Cell Death Discov. 2021).*

6) High abundant metabolites like salicylic and myristic acids (line438) interact with downregulated genes (line 439 and Fig. 5B). These results may be explained as the non-use and accumulation of these metabolites due to the low expression of the involved genes?

Author's response: We thank the reviewer for this observation and the likely explanation with which we agree. We found the interaction of the identified metabolites with the genes in the integrated interactome map. However, the interaction of metabolite salicylic acid and HIF1A is widely known in cancers (*McCarty MF et al., Practical strategies for suppressing hypoxia-inducible factor activity in cancer therapy. Med Hypotheses. 2010).* Similarly, The GUCA1A gene encodes for the GCAP1 protein that functionally regulates N-terminal myristoylation and lipid modifications in photoreceptors (*Hwang JY et al., Calcium- and myristoyl-dependent properties of guanylate cyclase-activating protein-1 and protein-2. Biochemistry. 2002)* and altered myristic acid accumulation can enhance fatty acid metabolism (*Rioux V et al., Regulation of mammalian desaturases by myristic acid: N-terminal myristoylation and other modulations. Biochim Biophys Acta. 2011).* Therefore, as the reviewer has correctly pointed out, the accumulation of these metabolites is due to the low production of enzymes/proteins as a direct consequence of low gene expression associated with the lack of Rb.

7) Figure S2B: "Correlation analysis followed by clustering showed the relationship between the Rb subtypes: advanced Rb group correlate positively with each other (red). Non-advanced Rb showed no correlation or negative correlation" This is not what is seen in the figure: Advanced and non-advanced groups showed similar results, correlation with each other but not with the normal retina where they showed no correlation or negative correlation

Author's response: We agree with the reviewer's suggestion and apologise for the confusion. We have made the necessary corrections. (Supplementary data, page 2, Figure S2B legend).

8. Validation of molecular signature that specify clinical and histopathological grades of Rb". Line 477/478 and Figure 6F, H: "RB1 and RBL2 expression was significantly low in bilateral Rb and groups D and E compared to pediatric controls". The results of these figures show that RB1 expression was significantly low in unilateral Rb but not in bilateral and in groups D and E, in these tumors the expression is not significantly low (Fig. 6F). Expression of RBL2 in bilateral, unilateral Rb and group D and E was significantly lower than in controls but the results show a great dispersion and it is difficult to see any difference with normal retina (Fig. 6H).

Author's response: We apologize for the confusing figure citation and agree with the reviewer's observation. We have made the necessary changes to the text. (page17, Results section, lines 489-495).

9. The statement in lines 533-537 about alteration in sphingolipid and branch-chain amino acids metabolisms, apoptosis and DNA repair between RB1 null cells and RB1 complemented cells, with reference to Figure S3D is not clear. In the legend of the figure is not stated how can be differentiated both types of cells.

Author's response: We agree with the reviewer's observation and have made necessary corrections to the figure number (page19, Results section, line 552.) The RB1 null and RB1 complemented cells are differentiated based on the RB1 gene expression and pRb protein expression apart from other molecular changes indicative of the same. The information is clarified in the figure legends as suggested by the reviewer (page20, Results section, lines 560-561).

10) Discussion, Lines 575, 680 and 588 the word amplification is not correct, expression should be used instead since the analyses determined RNA transcripts not the number of DNA copies

Author's response: As pointed out by the reviewer, we made necessary changes by replacing the word "amplification" with "expression" ( page21, Discussion section, lines 591, 596, 607).

11) Lines 585-586: The statement:"elevation of tumor-specific transcription factors like RUNX1, GABPB1, MYCN…their distinct transcriptional regulation in Rb tumors is a new observation" is not completely correct because MYCN amplification and expression in Rb tumors has been reported in many papers such as those of Gallie group in Toronto.

Author's response: The reviewer is absolutely right about this and we agree with the reviewer's point. We clarify that our study identifies RUNX1, GABPB1, and FOXO3 transcriptional regulation as new observations in advanced Rb groups. Although several MYCN studies on Rb have been reported, as pointed out by the reviewer, we demonstrate that within Rb clinical groups, MYCN expression is relatively higher in the advanced Rb tumors. We have made necessary changes to the text (page29, Discussion section, line 599-605)