Author's response to reviews

Title: Low penetrance of retinoblastoma for V654L mutation of the RB1 gene

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REVIEWERS’ COMMENTS:

Reviewer 1:

In this article, the author describe a mutation in RB1 gene associated with a low penetrance retinoblastome case. The following points need to be addressed:

1) The authors write in the abstract that the age of diagnosis varied from 16 and 46 years, but in results they write that the average age of the affected cases was 30 years old. They need to clarify this point. Retinoblastoma is diagnosed typically before the age of three years old. The are few cases published after five years old.

Response: Thank you for your detailed review. We apologize for the confusion. We revised the text to show that the four cases of retinoblastoma were diagnosed before the patients reached three years of age (2 y, 3 y, 5 m, and 1 m, respectively). After surgery, the four patients survived, and their ages now vary from 16 to 46 years (16, 45, 39, and 20 years, respectively). We revised our manuscript accordingly.

2) In methods, what are the primer sequences write in "polymerase chain reaction"?

Response: Thank you for pointing out our omission. We added the primer sequences in the ‘polymerase chain reaction’ section, as follows: “The coding region of the RB1 gene was amplified based on GeneBank NC_000013 as the reference sequence using specific forward (5’-atc ttt ccc agc ttg cat tt-3’) and reverse (5’-cat gat ttg aac cca gtc...
3) In conclusion, the last sentence is not correct. The V654L mutation is itself sufficient to develop low penetrance retinoblastoma. It is a typical mutation associated to low penetrance.

**Response:** Thank you for your comment. We changed our statement in the conclusion as follows: “This suggests that the RB1 V654L mutation is a typical mutation associated with low penetrance.”

4) In background, they write that low penetrance RB1 mutations are due "particularly from deletion of whole RB1 gene". This sentence is not correct.

**Response:** We revised the manuscript accordingly and deleted the phrase “particularly from deletion of the whole RB1 gene.” Thank you for your suggestion.

5) Results chapter is confusing. For example, the number of heterozygous germ-line mutation members are not clear.

**Response:** Thank you for your detailed review. We revised the results to clarify this issue as follows “Subsequently, the 29 family members (the members denoted by ‘#’ in Figure 1) of the index case consented to participate in this study. Comprehensive
mutations testing as well as retinoblastoma examinations were performed to identify both genotypes and phenotypes. Screening procedures in those at-risk family members showed that genotypes of 19 homozygous wild type (the members with # mark but without * mark) and an additional 10 heterozygous germ-line mutations (the members with # and * marks simultaneously in Figure 1) were identified and consented to participate in clinical examinations. Only four individuals, including the index case (two males and two females), developed unilateral retinoblastoma (the members in solid black), and seven asymptomatic subjects carry a germ-line mutation in one \textit{RB1} allele (the members with the slanted line).”

6) In discussion chapter, the authors do not comment anything about the cases described with V654L mutation.

\textbf{Response:} Thank you for your comment. We revised the first paragraph in the discussion to address why the penetrance of this mutation is so low in this particular family as follows: The \textit{RB1} V654L mutation is located on the B pocket domain of the pRB protein, which forms a functional repressor motif with the A pocket domain, important in the retina. Many missense mutations are located in the domains A and B affecting its structural folding and stability. Because valine and leucine are both hydrophobic, non-polar amino acids, the missense is not likely to have a significant
effect. The c.1960G>T mutation changes the final base of exon 19, and reduces the splicing score from 89 to 76.4 [13]. According to the previous study [14], the V654L mutation is in fact a splice mutation. The low-penetrance of c.1960G>C could result from the splicing mutation affecting only the last nucleotide of exon 19 [15]. The splicing machinery could alternate between the defective missense splicing (V654L mutation) and inactivation and skipping of exon 21 in the pocket box domain of RB1. This hypothesis could be why the penetrance of this mutation is so low in this particular family.

Reviewer 2:

The authors describe a large Taiwanese family with a mutation in the RB1 gene, which has in this family an extremely low penetrance. The mutation is found in 11 relatives, of whom only four develop retinoblastoma, at extremely high ages. This mutation has been described in other families, in which the penetrance was much higher. Therefore, the most interesting question is: why is the penetrance of this mutation so low in this particular family. However, the authors do not really answer this question, they only sum up what other authors have written as possible explanations why other RB1 mutations show low penetrance.
Major Compulsory Revisions

1) The manuscript should be edited to improve the use of the English language. As it is, it is at places hardly understandable what the authors mean.

Response: Thank you for your detailed review. The revised manuscript was edited by a native English speaking editor.

2) Because it is hardly believable that persons survive up to 46 years with untreated retinoblastoma, pathology reports or other proof that the tumors are really retinoblastoma should be added.

Response: Thank you for your detailed review. We apologize for the confusion. We revised the text to show that the four cases of retinoblastoma were diagnosed before the patients reached three years of age (2 y, 3 y, 5 m, and 1 m, respectively). After surgery, the four patients survived, and their ages now vary from 16 to 46 years (16, 45, 39, and 20 years, respectively). We revised our manuscript accordingly.

3) In the pedigree all family members tested should be marked, also those who do not show the mutation.

Response: Thank you for your suggestions. We revised the manuscript accordingly by adding the # mark to indicate the members who were tested, and * mark to indicate
those who have the mutation.

4) Given the late age at diagnosis, the age of mutation carriers without retinoblastoma should be given. The chance that these will develop retinoblastoma later on should be discussed.

**Response:** Thank you for your comment. We revised the results as follows:

“Furthermore, the ages of these seven mutation carriers without retinoblastoma are older than 20 years of age. We presume the chances that these family members will develop retinoblastoma later are low because retinoblastoma is rarely diagnosed after the age of 5 years. Only a few cases are thus published.”

5) The references are not properly chosen. e.g., Zhang et al., Hum Mutat. 29 (2008) 475-484 describes that the mutation V654L is in fact a splice mutation, this article is not mentioned. References 14-17 describe low penetrance RB mutations, but not extreme low penetrance. In the LOVD RB1 mutation database much more RB1 mutations are listed than in HGMD which is used by the authors.

**Response:** Thank you for your suggestions. We updated the references and database accordingly.
6) The authors should address the question: why is the penetrance of this mutation so low in this particular family.

**Response:** Thank you for your comment. We revised the first paragraph in the discussion to comment on the cases with V654L mutation and low penetrance as follows: The *RB1* V654L mutation is located on the B pocket domain of the pRB protein, which forms a functional repressor motif with the A pocket domain, important in the retina. Many missense mutations are located in the domains A and B affecting its structural folding and stability. Because valine and leucine are both hydrophobic, non-polar amino acids, the missense is not likely to have a significant effect. The c.1960G>T mutation changes the final base of exon 19, and reduces the splicing score from 89 to 76.4 [13]. According to the previous study [14], the V654L mutation is in fact a splice mutation. The low-penetrance of c.1960G>C could result from the splicing mutation affecting only the last nucleotide of exon 19 [15]. The splicing machinery could alternate between the defective missense splicing (V654L mutation) and inactivation and skipping of exon 21 in the pocket box domain of *RB1*. This hypothesis could be why the penetrance of this mutation is so low in this particular family.