August 24, 2006

Dear Editor of BMC Cancer:

Please, receive herein the revised online version of our manuscript nº 9541640910666569 entitled: Frequent promoter hypermethylation of RASSF1A and CASP8 in neuroblastoma, by Lázcoz et al.

I include a point by point list of explanations to the reviewers, in order to satisfy their requirements for publication. Please, also note that professionals of English editing for scientific publications (BioScience Editing, an Internet company) have done an extensive editing of the manuscript.

Yours sincerely,

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Answering to reviewer Yoshitaka Sekido

Major compulsory revisions

1. One of the most critical points of this paper is that the methylation status of each gene is not correlated with its mRNA expression except for RASSF1A. From this standpoint, the authors’ conclusion is very ambiguous if hypermethylation of the three genes, BLU1, NORE, and CASP8, is important as an epigenetic alteration in the development of neuroblastoma.

Answer: We have totally changed the conclusions to follow the reviewer’s suggestion.

2. The authors showed that there was just a statistical association between the hypermethylation status of RASSF1A and CASP8 in 12 cell lines. However, there seems to be a very weak biological relationship between two gene products, and epigenetic modifications of these genes seem more likely to be independent, and probably due to coincidental events. Therefore, the title of this paper should be rephrased more appropriately. For example: Frequent promoter hypermethylation of RASSF1A and CASP8 in neuroblastoma.

Answer: We have changed the title to the one suggested by the reviewer.

3. The authors should provide the data on MYCN amplification, 1p deletion and the aggressive histological pattern of the tumor by making a new table or incorporating the data in Table 3. If the data are not given in detail, the authors should delete their statement about this from the Abstract.

Answer: We have added a new table (Table 1) containing these data.

4. The authors’ conclusion, LOH and MSI at 3p21 may be responsible for tumor formation in a small number of neuroblastic tumors, seems unclear. Although the low frequency of LOH at 3p may indicate that a candidate tumor suppressor gene of neuroblastic tumors is not located in this region, MSI at chromosome 3p does not seem to indicate any clear scientific meaning. Since MSI is not usually limited to a specific chromosomal region, which usually implies a phenotype over a whole genome, this conclusion should be rephrased.

Answer: we have changed the conclusion, reinforcing the fact that a candidate tumor suppressor gene of neuroblastic tumors is not located in this region.

5. In Table 3, the term, n.d. (not determined), for LOH/MSI data is quite confusing. Does it mean non-informative?

Answer: It indicates that a particular case was not studied due to lack of blood to be paired with the tumor sample.

6. There seems to be a discrepancy between Table 3 and Figure 1 about the results of D3S3522. Although the data of #48 look like no LOH/MSI, and the one of #77 shows a deletion of one band among three, the results are just described n.a. and -, respectively, in Table 3. The authors should carefully check the data again.
Answer: We have made the following corrections:
- Table 3: patient 48 LOH study with marker D3S3522 produces a ‘−’ result instead of a ‘n.a.’ Patient 77 produces LOH for the same study, instead of ‘−’.
- Legend to figure 1 has been changed accordingly.
- Along the text we have made corrections on the frequency of LOH.

7. In Figure 2B, C, and D, the authors should give the patient’s number for each lane to be compared with the data in Table 3.

Answer: We have corrected those numbers in Figure 2.

8. The authors should indicate the definition of hypermethylation and hemimethylation.

Answer: It has been defined at the beginning of the Discussion (first paragraph).

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Page 6 lines 17: How did the authors measure the 75 ng of cDNA that was synthesized from RNA with reverse-transcriptase?

Answer: We retrotranscribed 1 ug of RNA from the control and from the cell lines. Knowing the efficiency of the enzyme we can approximately calculate the amount of cDNA we obtain. We can also measure the concentration by spectrometry.
Answering to reviewer Reinhard Dammann

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

- It would be interesting to include more findings on the correlation between methylation status and clinico- or histopathological data.

Answer: We consider the results already presented in the manuscript are the only one that can be documented. No more findings can be introduced. We have, nevertheless, included a new table with clinicopathological and genetic data of the neuroblastic tumors.

- It would be interesting to analyze the methylation of the four genes in the 21 blood controls.

Answer: We would have liked to do the methylation analysis even in adjacent normal tissues to the tumors of the different patients. This is how most scientists do, whenever possible. In our case, we did not have those tissues, and considered that evaluation in blood might give misinterpretation, as methylation is usually tissue specific.

- The expression data of Nore1 are not convincing. What means low expression? It would be helpful to perform real-time RTPCR or to reactivate promoters by DNA methyltransferase inhibitors.

Answer: Low expression means a lower level of expression respect to the normal control. When we do retrotranscription, starting from the same amount of RNA in the control and in the cell lines, the expression can be semiquantitated, comparing the intensities of the PCR bands.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

- The conclusion that RASSF1A methylation might be an alternative mechanism to alter the Ras signaling pathway in neuroblastoma is clearly an over-interpretation of their data: the authors have not analyzed Ras mutations in their samples.

Answer: We have deleted this conclusion.

- The writing is not acceptable. The abstract is poorly written and the number of analyzed cases is missing. The discussion is weak. The quality of written English is nor suitable for publication.

Answer: professionals of English editing for scientific publications (BioScience Editing, an Internet company) have done an extensive editing of the manuscript. The abstract has been modified in its English style, and by the incorporation of new conclusions and the number of cases analyzed in the study. The Discussion now presents a new paragraph (the first one), to better explain the ways of tumor suppressor gene loss of function when methylation takes part as an epigenetic phenomenon.