Reviewer's report

Title: Expression of ZIC family genes in meningiomas and other brain tumors

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Reviewer: Irene Szijan

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The expression of ZIC family genes in meningiomas was studied at: i) mRNA level, by RT-PCR in 3 meningioma specimens and ii) Protein level, by immunohistochemistry on 22 grade I, one grade II and one grade III meningiomas.

mRNA levels of ZIC1, ZIC2 and ZIC5 in the 3 meningioma samples were higher than in whole brain but not as high as in medulloblastoma. Expression of ZIC1 in grade II meningioma was significantly higher than in grade I (data from public microarray).

Immunohistochemical analysis of different meningioma types using anti-ZIC antibodies revealed positivity to ZIC1/ZIC2/ZIC5. Similar results were obtained in arachnoid cells of normal human meninges. ZIC proteins were analyzed in developing mice showing that they are expressed in meningeal cell progenitors.

The conclusion is that ZIC proteins are possibly involved in the proliferation of meningioma cells. The authors consider that the major finding is the differential expression of the 5 ZIC genes in brain tumors: ZIC1/ ZIC2/ ZIC5 are expressed in meningiomas while all ZIC family including ZIC4 are expressed in medulloblastoma.

The main objection is the small number of samples used to estimate ZIC mRNA levels, 3 meningioma specimens and, in addition, one of them expressed ZIC4. On the other hand, immunohistochemical analysis revealed negativity for ZIC4 in all meningioma grades.

Summarizing, the objectives are clear and an appropriate methodology was used. Results are interesting but there are some mistakes and lack of details in describing them. The discussion should be more extensive in the analysis of results and conclusions are not elaborate enough with regard to the significance of results obtained. Therefore, the manuscript needs some revision as follows:

Major Compulsory Revisions

1) Results: Expression of ZIC1-5 at the mRNA level by RT-PCR analysis was studied in 3 meningioma specimens. This is an insufficient sample size, not enough for definite conclusions as the results obtained may only suggest a hypothesis. On the other hand, the immunohistochemical analysis of these proteins shows their presence of them in 24 meningioma samples which may support the RT-PCR results. This possibility should be mentioned.
2) The data in Figure 1B do not agree with those in the text: In the figure, ZIC3 is expressed in the 3 meningiomas and ZIC5 in one of them, contrary to what is stated under Results (pages 9 and 10).

3) Data about normalized transcript levels in meningiomas in Figure 1B do not tally with the respective band-intensity in Figure 1A, which is far greater for ZIC1 than for ZIC2 and very tiny for ZIC5.

4) Results, line 4: Semiquantitative RT-PCR: Its determination should be explained.

5) Figure 6 is too crowded and the legend is confusing. It would be better to describe the abbreviations/probes/staining/colors and all other explanations in every set of sections, which display results of the same tissue or developing stage. The sections could be grouped as for example, A,B,E,F (meningeal membrane); C,D,G,H (cerebellum); I,J,M,N,O (meningeal cells in developing mice) and L,P (mature arachnoid cells).

6) Figure 6H: It is not clear whether a cytoplasmic staining of the Purkinje cells indicated by arrowheads is really that.

7) Discussion, page 13, 1st paragraph, line 3: The statement “we considere it possible that ZIC proteins are involved in the proliferation of meningioma cells. This is consistent with our observation that ZIC1 mRNA levels are higher in Grade II than in Grade I tumors” should be supported by results showing a higher level of ZIC expression in meningiomas in comparison to normal tissue but not by a higher expression in grade II than grade I meningiomas.

8) Page 14, 2nd paragraph: the statement about the presence of ZIC4 in paraneoplastic syndrome, small-cell lung cancer and other tumors should be discussed more extensively, taking into account its expression and that of other ZIC proteins in more/less aggressive tumors or syndromes.

9) Conclusions: In Point 1 it is necessary to add that the expression is referred to mRNA levels in order to differentiate this statement from that in Point 4.

Minor Essential Revisions:

1) Figure 2: the mRNA level of ZIC1 in grade II tumor is 5 fold that of normal dura mater, not 5.4 fold.

2) Methods, page 6, 2nd paragraph: The statement “RNA samples were prepared using TRIZOL…” should describe which samples. Are there from human brain tumors?; Line 15: The term ZIC3_R should be deleted.

3) Results, page 11, the last sentence of the 1st paragraph should include the number of the table containing the data.

4) Page 11, second paragraph, line 5: “CX32/ZC26-positive signals were detected…” is stated in the text, but ZC26 fail to appear in Figures 4 and 5; In the same paragraph “data not shown” in brackets: what data do they refer to?

5) Page 12, second paragraph, line 4: “CX32 positive signals were detected in arachnoid cells…but not in the dura mater”, the characters “a” and “d” displayed
in figure 6B should be indicated in the legend to this figure.

6) Figure 6K, O: The function of CS56 staining should be explained.

7) Legend to figure 6, page 21, line 6 from bottom: “The positive signals were detected in the aracnoid, pia mater and perivascular cells (P)”. In this figure the pia mater is not indicated.

8) Discussion, page 13, 2nd paragraph, line 5: The sentence “We demonstrated that the presence of Zic/ZIC proteins in meningeal cells is conserved between mouse and human”; It should be explained how this was done. Is it doe to mouse Zic antibody recognition of human ZIC proteins?

9) Abstract, Results, 2nd line: data about ZIC transcript levels in normal dura mater are not shown.

Discretionary Revisions:

1) Results, page 11, line 9: It is not clear if the epitope-tagged ZIC4 protein does not show an obvious cytoplasmic localization (Supplemental Figure 1).

2) Results, page 11, line 4: The statement about staining intensity and distribution across tissues of signals for antibodies CXY2 and ZC26 should include “Data not shown”.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests