Author's response to reviews

Title: Polysialic acid is associated with better prognosis and IDH1-mutation in diffusely infiltrating astrocytomas.

Authors:

Katri Mäkelä (katri.s.makela@uta.fi)
Kristiina Nordfors (kristiina.nordfors@gmail.com)
Jukka Finne (jukka.finne@helsinki.fi)
Anne Jokilammi (anne.jokilammi@utu.fi)
Timo Paavonen (timopaavonen@uta.fi)
Hannu Haapasalo (hannu.haapasalo@fimlab.fi)
Miikka Korja (mkorja@mneurosurgery.com)
Joonas Haapasalo (joonas.haapasalo@gmail.com)

Version: 2 Date: 30 June 2014

Author's response to reviews: see over
Point-by-point responses to the Reviewers’ reports

Dear Editor,

We hereby submit the manuscript for re-evaluation. Please find below the point-by-point responses to the questions and concerns that the Reviewers have presented. The corrections made in the manuscript have been highlighted in yellow color.

While the manuscript was under review, we were able to update the study material, mainly in form of adding new information to the patient follow up study. Therefore some of the results have changed. Furthermore, we were delighted to find that polysialic acid was an independent prognostic factor in the Cox multivariate survival analysis, which was suggested to be done by the reviewers. These new findings are in line with our previous hypothesis and study findings. They are discussed more thoroughly in the answers to the reviewers and also in the manuscript. We hope that the improved manuscript is suitable for publication in BMC Cancer.

Sincerely,

Katri Mäkelä, corresponding author

Reviewer's report # 1:

The authors reported that PolySia positivity is associated with longer survival in glioma and its relation with IDH1 mutation. The concept is simple and easily understandable. However, the data addressed are currently very limited in scope. If authors address the following comments, this manuscript could be strengthened.

1. The authors should perform multivariate analysis including age KPS, IDH1 mutation, EGFR amplification, MIB1 labeling index, polySia expression, and NCAM expression.

The multivariate survival analysis was redone as the reviewer recommended. However, unfortunately we were not able to include the KPS into the new analysis because it has not been followed and recorded into the data of the study material. All the other variables inquired were available to the analysis. Also, during the time the original manuscript was under review, we updated the survival data of the study material, and new results are presented below.

The following variables were taken into the new multivariate survival analysis: patient age, IDH1 mutation, EGFR amplification, MIB1 labeling index, polySia expression, NCAM expression and WHO grade. There were 77 cases in which all these variables were known. These 77 cases were included in the Cox multivariate analysis. The analysis was not done separately in each WHO grade because the cases included would have been few. IDH1 mutation, patient age, MIB1 and polySia came up as independent prognosticators. The hazard ratio (Exp(b)) for IDH1 was 0.195 (95%CI for Exp(B) 0.101-0.376). Exp(B) for patient age was 1.705 (95%CI for Exp(B) 1.217-2.390). Exp(b) for MIB1 was 1.673 (95% CI for Exp(B) 1.210-2.312). Exp(B) for polySia was 0.516 (95% CI for Exp(B) was 0.280-0.951). Age cut off points were 0-54, 55-69, 70-. Cut off points for MIB1 were 0-5, 5.1-15, 15.1-.
Added to results (Page 8, paragraph 2, line 1): Cox multivariate survival analysis was done with the following variables: patient age, IDH1 mutation, EGFR amplification, MIB1 labeling index, polySia expression, NCAM expression and WHO grade. There were 77 cases in which all these variables were known. The analysis was not done separately in each WHO grade because the cases included would have been few. IDH1 mutation, patient age, MIB1 and polySia expression came up as independent prognosticators. The hazard ratio (Exp(b)) for IDH1 was 0.195 (95%CI for Exp(B) 0.101-0.376). Exp(B) for patient age was 1.705 (95%CI for Exp(B) 1.217-2.390). Exp(b) for MIB1 was 1.673 (95% CI for Exp(B) 1.210-2.312). Exp(B) for polySia was 0.516 (95% CI for Exp(B) was 0.280-0.951). Age cut off points were 0-54, 55-69, 70- . Cut off points for MIB1 were 0-5, 5.1-15 , 15.1-.  

Added to discussion (Page 9, paragraph 1, line 4): In multivariate survival analysis polySia came up as an independent prognostic factor, indicating better prognosis  

Added to results (page 7, paragraph 3, line 1): The median follow up time of survivors was 133 months.

2. The authors should show the data of grade III-IV glioma but not grade II-IV glioma. Patient number should be indicated in Kaplan Meir data. Detailed histological diagnosis should be indicated.  

Patient survival by polySia expression was studied also within different grades. The results were significant when all grade II-IV tumors were included, but also when grade IV tumors were studied separately. Significant correlation to patient survival was also found, when grade III and IV tumors were studied as one tumor entity. However, no significant correlation was found when grade II and III tumors were studied separately. NCAM expression associated significantly with patient survival in the total tumor material as well as when grade III and IV tumors were studied simultaneously. No significant association was found within grade II or III tumors, when studied separately.  

Patient numbers are added to the Kaplan Meier data in the Figure legends.  

Table 1 has been updated to whole tumor material  

Detailed histological data on grade IV tumors was known. There were 1 giant cell glioblastoma, 10 gliosarcomas and 170 of the grade IV tumors were glioblastomas.  

Added to M&M (page 4, paragraph 3, line 2): Of the grade IV astrocytomas, 10 were gliosarcomas, 1 was a giant cell glioblastoma and 170 were glioblastomas.
Reviewer's report # 2:

In this brief manuscript, the authors aimed to characterize the expression of NCAM, polySia, and polySia-NCAM in a range of gliomas and to determine whether polySia-NCAM could be of additional prognostic utility in grade II-IV astrocytomas. They found that polySia and NCAM are frequently expressed, and that PolySia is associated with IDH mutation and better prognosis.

Major compulsory revisions:

1. Please describe how samples were scored for positivity or negativity for polySia and NCAM. (For example, if only one cell in the core is positive, was this tumor scored as positive? 10% of cells? Majority?)

The TMA samples were scored polySia-positive, if the expression was detected at least in 30% of countable cells in the confocal microscopy.

2. Among grade II-IV gliomas, the reviewers found that polySia was not associated with WHO grade, yet they found that polySia-NCAM was associated with IDH1 mutation. Because it is known that IDH1 mutation characterizes grade II and III diffuse gliomas and secondary GBM, but is not a feature of grade I gliomas or primary GBM, how do the authors explain the lack of association with grade?

Of the total tumor material 181 were grade IV tumors, and of these, IDH1 mutation status was known of 145 tumors. Of these 145 grade IV tumors, only 19 tumors were IDH1 positive. As the reviewer also presents, IDH1 mutation occurs more often in grade II and III tumors than in grade IV tumors, oppositely as polySia expression. We performed additional statistical analysis in the whole tumor material to assess the secondary tumors, as well (previous analysis considering grade were done only in primary tumors. Importantly, in the whole tumor material including secondary tumors there was no association between polysia and grade. Also, we tested if there was a difference in polySia expression between primary and secondary glioblastomas, no significant difference was found. The dividing of glioblastomas in two genetically different subset of groups (primary and secondary GBMs), may be a confounding factor in this matter. We thank reviewer for raise this important issue and think that the analysis made in the whole tumor material are more reliable.

3. Similarly, the authors found positive polySia expression to be associated with increasing proliferation as determined by Ki-67 staining, yet they also found that polySia expression was associated with better prognosis. The authors touch on this point briefly, but should expand on these seemingly discordant findings. (The latter association with better prognosis would be consistent with their finding of polySia association with IDH mutation, but the Ki-67 finding is unexpected.)

PolySia is clearly a feature of malignant tumors, as also shown in previous studies, which have also been referred in the manuscript (Amureux et al., 2010 and Petridis et al., 2009). However it seems that amongst these malignant tumors, patients with polySia expressing tumors have better prognosis than those whose tumor is polySia negative. The situation could be compared to IDH1. IDH1 mutation is also a feature of malignant tumors, but yet it is well known to be a marker of better prognosis amongst these tumors.
The association was studied in the entire tumor material, consisting all grade II-IV tumors. The association was not significant when studied in different grades. Cox multivariate analysis polySia came up as an independent prognostic factor and thus was not connected to Ki-67/MIB-1. We can state that the mechanisms in protein expressions of the cells are complex and widely unknown. This question is thus difficult to answer currently without further studies on the subject.

Added to Discussion (page 9, paragraph 1, line 8): Although polySia was associated with better prognosis in univariate and multivariate analysis, it was also associated with increasing proliferation. This underlines that mechanisms in protein expressions are complex.

4. Please specifically state whether polySia-NCAM is still prognostically meaningful after controlling for IDH1 mutation and patient age. Is the survival effect of polysia-NCAM purely a function of its association with IDH1 mutation? Is the claimed utility of polySia-NCAM expression as a new additional prognostic factor for gliomas additional to the prognostic information that can already be inferred from IDH1 status? The authors did make the survival curves looking at “both positive,” “one positive” and “both negative”, however that analysis does not clearly address the question because one cannot discern in the ‘one positive’ group what the effect of is of the individual PSA or IDH positives.

We agree with the reviewer that the survival curves, looking at “both positive,” “one positive” and “both negative”, are problematic. Therefore we have decided to omit this analysis from the manuscript. Also the figures 4 and 5 presenting the Kaplan Meier curves of this analysis have been omitted.

Cox multivariate survival analysis was redone after update of the study material. PolySia came up as an independent prognostic factor. This indicates that the survival effect is not a function of its association with IDH1 (Results page 8, paragraph 2, line 1).

5. The antibodies for NCAM, PSA, and polySia-binding fusion protein is not adequately described in the methods section. Please explicitly describe the source and conditions, at least briefly.

We have written in the Methods section “Confocal microscopy was performed as described earlier [16].” In brief, polySia-binding fluorescent fusion protein (EndoNA2-GFP) at a concentration of 10 μg/ml was used for polySia detection as described earlier [Jokilammi A, Ollikka P, Korja M, Jakobsson E, Loimaranta V, Haataja S, Hirvonen H, Finne J: Construction of antibody mimics from a noncatalytic enzyme-detection of polysialic acid. J Immunol Methods 2004, 295(1–2):149-160]. Two hundred microliters of 4 μg/ml fusion protein in PBS was added to the cells. The cells were incubated for 1 h at room temperature and washed six times with the buffer, counterstained with DAPI (Molecular Probes) as described by the manufacturer, washed four times with the buffer and mounted on object glasses with Immu-mount. Mouse anti-human NCAM antibody (123C3) at a concentration of 4 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a primary antibody. Immunohistochemical incubations were done overnight at 4°C. In immunofluorescence, Alexa Fluor 594 chicken anti-mouse secondary antibodies (Molecular Probes, Eugene, OR) were used, and slides were mounted with Immu-Mount (Shandon, USA).

Added to M&M (page 5, paragraph 2, line 1): PolySia-binding fluorescent fusion protein (EndoNA2-GFP) at a concentration of 10 μg/ml was used for polySia detection. Mouse anti-human NCAM antibody (123C3) at a concentration of 4 μg/ml (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a primary antibody. Immunohistochemical incubations were done overnight at 4°C. In immunofluorescence, Alexa Fluor 594
chicken anti-mouse secondary antibodies (Molecular Probes, Eugene, OR) were used, and slides were mounted with Immu-Mount (Shandon, USA).

**Minor Essential Revisions:**

6. The scale bar and lettering on Figure 1 is very difficult to read.

The scale bar and lettering of Figure 1 has been changed and is now easier to read.

**Discretionary revisions:**

7. In their characterization of staining, the authors classify tumors as positive or negative, and show only images from one positive and one negative tumor. However, as the authors emphasize that polySia-NCAM is a marker of stem cells, it would be informative and would elevate the paper to know whether polySia and NCAM colocalizes with specific cellular subpopulations within the tumors (e.g. in cells positive for stem cell markers or proliferative markers).

We thank the reviewer for this interesting point. Unfortunately we don’t have an opportunity to study the subpopulations of the tumors within the current study material. However, it could be a theme of a study in future. This interesting point is added to the discussion of the manuscript.

Added to discussion (page 11, paragraph 1, line 3): Also, since polySia-NCAM have been proposed to be marker of stem cells, another interesting theme for future research would be to study the expression of polySia and NCAM and their colocalisation in specific cellular subpopulations of diffusely infiltrating astrocytomas.
Reviewer's report # 3:

This is a well written manuscript which is worthy of publication. There is, however, one major compulsory revision:

1. The authors finding that polysialic acid expression is a good prognostic feature in astrocytic neoplasms grades 2-4. This is at variance with several other studies referenced by the authors. Because this is a critical finding of this study, the factors that lead to a different conclusion from previous studies should bediscussed in detail.

There are two studies that we have referred in the manuscript, in which polysialic acid expression has been connected to worsened patient outcome. These are Petritdis et al., 2009 and Amoureux et al., 2010. The study of Petridis et al. and their research frame has already been discussed in our manuscript.

Amoureux et al. found a correlation between polySia and worsened patient outcome. They had included 56 glioblastomas to their study and no other grade astrocytomas. The study frame differed from ours, since the main method to detect polySia expression was an enzyme linked immunosorbent assay (ELISA). Immunohistochemistry was also performed and the expression was measured semiquantitatively. The results acquired by immunohistochemistry and ELISA test were tested and found to correlate with each other. Thus the final survival analyses were done with the ELISA test data. When compared to our study, the studymaterial of Amoureux et al. is smaller and includes only 56 GBMs, here we have 242 grade II – IV astrocytomas and 146 primary glioblastomas. Furthermore, the mean follow up of the survivors by Amoureux et al. was 8 years. In our material the same follow up time is 11 years. Thus we think that our data of patient material is more profound. It can also be questioned if utilisation of the ELISA test data has affected the survival data results. They did not show survival data analysis on immunohistochemistry results. Here we show the anatomical results with confocal microscopy and verify that the polysialic acid expression was linked to NCAM expression. In summary, the dissimilarity of the study material and study frame may explain the difference in the study findings between our study and the study of Amoureux et al.

Added to discussion (page 10, paragraph 1, line 1): Previously, Petridis et al. [9]as well as Amoureux et al.[10], have presented correlations between polySia expression and tumor malignancy in astrocytomas

Added to discussion (page 10, paragraph 1, line 10): In this study, larger patient population, longer patient follow up time, confocal microscopy describing the anatomical location, and importantly, assessment of IDH mutation might provide additional value to the analysis.