Author's response to reviews

Title: Carbonic anhydrase IX in oligodendrogial brain tumors

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Author's response to reviews:

Dear Reviewers,

thank you for your valuable and professional comments. We truly appreciate your efforts and we have now improved our manuscript based on your questions. Below you will find specific answers to each and every question you pointed out, as well as place where this improvement is in the manuscript. We hope that you will find these improvements satisfactory and find our manuscript interesting and relevant.

Sincerely, Sally Järvelä

Reviewer Vordermark.

Major Revisions

1. Page 6, Materials and Methods, 1st paragraph, survival data. The reviewer correctly pointed out that there were some inconsistencies in the percentage values for the survival rates. We apologize this error, and we have corrected the sentence.

Page 6, line 8.

- The median follow-up time was 3.5 years (mean 4.9, SD 4.5). During the follow-up, 43 patients died.
2. Page 8, 2nd paragraph, CA IX groups and Fig.2. In the previous Figure 2, completely negative tumors were compared to those which expressed CA IX to some extent. This was mentioned in the Results section, where the survival data is presented. However, we realized that the Figure 2 was not clear in this aspect, because of the CA IX - and CA IX + markings. We have modified the picture and markings to make the interpretation easier, and we also added a sentence that should further clarify this aspect in the right context.

(Page 11, 1st paragraph.

- The analyses were done by comparing CA IX groups based on the immunostaining reactivity. When stated, CA IX negative group was compared to CA IX positive group.

In survival analysis overall survival was defined from the day of surgery until the death of the patient. The death of patient was considered as an event in the analysis.

Univariate survival analysis was carried out by comparing all the four CA IX categories. In multivariate survival analysis the tumors that had no CA IX immunostaining were compared to those which had weak, moderate or strong staining. This was done to reach the best cut-off possible.

3. Pages 10 and 12, survival analysis. We analyzed overall survival. A death of the patient was considered an event in Kaplan-Meier analysis. The variables used in the multivariate analysis are listed in the Results.

Page 13, line 7.

- In Cox multivariate analysis the expression of CA IX, patient age and histological component (pure oligodendroglioma vs. mixed oligoastrocytoma) showed independent prognostic significance (p=0.009, Exp(B)=7.370; p=0.003, Exp(B)=3.422 and p=0.022, Exp(B)= 0.351). CA IX positivity, older age and astrocytic component predicted poorer outcome. Other clinicopathological features, including proliferation status and 1p 19q status, did not reach statistical significance in association to the prognosis.

4. Discussion, concentrating on CA IX in brain tumors and dealing with HIF-1 alpha expression.

As suggested by the reviewer, we have added the following information.

Page 16, line 8:

- A recent study by Birner et al (Birner et al, Expression of Hypoxia-related
Clinical Cancer Research (2004) in 60 oligodendroglial tumors with 1p aberrations showed that CA IX expression is often accompanied with the expression of hypoxia-inducible factor 1α (HIF-1α). They concluded that when expressed together, they represent a true tissue hypoxia and not just oncogenic activation, which might otherwise be the case, at least for HIF-1α. It is already known that HIF-1α is one of the key factors regulating cellular O2 homeostasis and its activation represent a key step in angiogenesis and adaptation to hypoxia and thus the vitality of the tumor. Overexpression of HIF-1α is shown to be an independent prognostic factor in oligodendroglial tumors and interestingly, it also correlates with the microvessel density in these tumors (Birner et al, Expression of Hypoxia-Inducible Factor-1α in Oligodendrogliomas, Cancer 2001). They suggested that as tissue hypoxia is known to diminish the efficacy of radiotherapy and thus influence the adjuvant therapy given to the patients, the evaluation of tissue hypoxia using this combination of hypoxia-markers would be helpful for recruitment of patients for individualized therapy strategies, e.g. identification of hypoxic tumors for hyperbaric oxygenation preceding radiotherapy.

Furthermore, the basic reaction of MnSOD where reactive oxygen species superoxide O2- is converted to H2O2 by MnSOD is known to affect to HIF-1α, by letting it accumulate into the cytosol. Thus, MnSOD, HIF-1α and CA IX expressions are representing an exciting and complicated continuum.

5. The quality of Figure 1. We have now improved the Figure 1 and hope that it is now acceptable for publication.

6. The problems of Figure 2. As suggested by the reviewer, we have made Figure 2 simpler and easier to read.

7. Table 1. Table 1 is now inserted to the text file!

Minor Revisions

1. Page 3, Introduction, 1st paragraph. Our study is a multicenter study and based on archival material collected in 1980-2004. Unfortunately this means that we have not been able to get all the precise treatment data from all the patients. However, in Tampere University Hospital the normal practice is that astrocytic tumors are treated with radical resection and radiation therapy, sometimes temozolomide is used as adjuvant therapy (during the 60Gy radiation and after that 6 courses in four week intervals), especially in GBMs if the patient condition allows this. In grade III astrocytomas the benefits of adjuvant chemotherapy is not so clear, so it is considered from case to case. In oligodendrogliomas resection is also done, but after that, chemotherapy is used more often and radiation therapy is used only after consideration. The patients with
oligodendroglioma seem to benefit from chemotherapy, especially if they have LOH1p19q. PCV or temozolomide are commonly used, but CCNU alone or combined with following temozolomide course can be used, as well as carboplatin, taxanes etc. Nevertheless, I have to admit that the procedures in the treatment of gliomas are varying so much even within one country. Therefore, we decided to change the sentence.

Page 3, line 5.
- Accurate diagnosis is important in the case of oligodendrogliomas because the pathophysiology, treatment options and prognosis vary from that of diffusely infiltrating astrocytomas (1).

2. Table 1. The percentage numbers are now in the correct place.

Reviewer Preusser

Major Revisions

1. CA IX and AOE categories. The categories are based on the reactivity of the staining taking equally into account both the intensity and extent of the staining. There were some problems while packing the image-files that lowered the quality of the photographs. Therefore, we have improved the Figure 1. Immunostaining results were evaluated by three observers during one session on a multiheaded microscope. A clear consensus was reached with each tumor specimen regarding to the staining reactivity. Because of this research method we can not provide kappa values. Based on our extensive experience with CA IX immunostaining in various tumor types we are completely sure that the evaluation reached good consistency.

Page 8, line 6.
- The immunostaining results were evaluated by three observers (HH, YS, SJ) semiquantitatively by dividing the CA IX and AOE staining reaction into four categories based on the reactivity of the staining taking equally into account both the intensity and extent of the staining.

2. CA IX immunolabelling. The signal for CA IX is both membrane-associated and intracellular in oligodendroglial tumors. Perinecrotic accentuation was expressed in grade III tumors and the staining was often focal in other areas and grade II tumors.

Page 8, line 14.
- The immunostaining of AOE s has been described in more detail in our
previous study (10). Briefly, all the AOEs (MnSOD, GLCL-C, GLCL-R, Trx, TrxR) were expressed diffusely in the sections. MnSOD had granular and TrxR perinuclear cytoplasmic immunoreactivity and rest of the AOEs were stained diffusely in the cytoplasm.

The signal for CA IX is both membrane-associated and intracellular in oligodendroglial tumors. CA IX expression mainly showed focal accentuation and was found to be most intense in perinecrotic areas. When necrotic or microvascular proliferation was present, perinecrotic or microvessel staining was commonly seen.

The CA IX and AOE stainings represent the same area of the tumor because the adjacent sections of the same tissue micro-array block were used in the analysis.

3. Correlation between CA IX-positivity and presence of necrosis and microvascular proliferation. When necrotic areas were present (in grade III oligodendrogliomas) perinecrotic expression of CA IX was seen. Furthermore, it can be said that the most intense staining was found in perinecrotic areas. Also, in addition to the expression in the cancerous cells, a clear expression of CA IX that associated with microvascular proliferation (endothelial cells expressed CA IX strongly in the capillaries and arterioles) was seen in few samples. This was particularly true in those samples that had very prominent, but quite irregular vascularization.

See the question 2 and improvement based on that. In addition we, added text into the discussion concerning this phenomenon.

Page 15, line 20.

- In addition, both in astrocytic brain tumors and in oligodendrogliomas perinecrotic accentuation is quite commonly seen. A study by Preusser et al (41) shows that CA IX has a positive correlation with the presence of necrosis. When necrosis or microvascular proliferation was present, perinecrotic or microvessel staining was commonly seen in our present study, too. This could reflect the severe changes disturbing the tissue (hypoxia, insufficient microvasculature etc.) leading finally to necrosis but also enhancing the expression of CA IX.

4. Proliferation activity details. When proliferation activity was assessed, the hot spot with highest density of immunolabelled nuclei was analyzed. This information is now added into the methods-section.

Page 9, line 8.
Briefly, the analysis was done on areas that were expressing quantitatively the highest number of immunopositive nuclei. Twenty microscopic fields (×400 magnification) were counted along a vertical and horizontal axis perpendicular to each other. Endothelial cells, necrotic and haemorrhagic areas as well as section borders were omitted.

5. Proliferation index observation. We have done careful analysis regarding MIB1 proliferation index and we are confident that our results are reliable and well-repeatable. We would like to refer to a previous study from our research group: Sallinen et al in Prognostication of astrocytoma patient survival by Ki-67 (MIB-1), PCNA, and S-phase fraction using archival paraffin-embedded samples. reported in J Pathol. 1994 Dec;174(4):275-82. We added information into the methods-section, as well as discussed this phenomenon in the discussion section.

Page 18, line 1.

We also found an association between CA IX and proliferation index (MIB-1). In our material, CA IX(+) tumors had significantly lower proliferation index than their CA IX(-) counterparts. This could reflect the severe hypoxia that is present in the tumor tissue, thus affecting to cells capability to proliferate. A study by Proescholdt et al (Proescholdt et al, Expression of hypoxia-inducible carbonic anhydrases in brain tumors, Neuro Oncol 2005) shows that the relationship between CA IX and proliferation index is quite complicated. They examined the expression of CA IX and CA XII in different kind of brain tumors and also in the normal brain. They found an association between CA IX positivity and increasing proliferation index. However, their material was quite heterogeneous, and oligodendroglialomas were not included at all. Despite of that, it can be said that the correlation between CA IX and proliferation is interesting and should be studied more thoroughly.

6. TUNEL stained sections details. We added details into the methods-section.

Page 9, line 20.

Staining was analyzed by counting all the tumor cells in the core tissue with an image analysis system (CAS-200). The obtained scores were reported as a percentage of immunopositive nuclei.

7. MnSOD and CA IX spatial relationship. The question of the spatial relationship is very interesting. However, we regret that for now, it is not possible to perform double immunolabelling. The MnSOD analysis has been made previously and we are now lacking the antibody (that was a gift from another
laboratory). However, we believe that our results reflect the real situation. On tissue micro-array technique the sections are very thin (6 um) and they are sequential sections of the same tumor region, thus the distance of these two sections is very small. In this case, only the type of expression was different; MnSOD is expressed more diffusely and granularly, while CA IX expression was more focal and cytoplasmic. We ask you kindly to check the pictures from our previous article, where MnSOD expression is described in more detail: Järvelä S et al. Antioxidant enzymes in oligodendroglial brain tumors: association with proliferation, apoptotic activity and survival. J Neurooncol. 2006, 77(2):131-140.

Page 17, line 19.

Furthermore, the basic reaction of MnSOD where reactive oxygen species superoxide O2- is converted to H2O2 by MnSOD is known to affect to HIF-1¿, by letting it accumulate into the cytosol. Thus, MnSOD, HIF-1¿ and CA IX expressions are representing an exciting and complicated continuum.

Minor Revisions

1. Prognostic data ¿ oligodendrogliomas vs. oligoastrocytomas. Pure oligodendrogliomas had more favorable prognosis. This is now added into the text.

Page 13, line 7.

In Cox multivariate analysis the expression of CA IX, patient age and histological component (pure oligodendroglioma vs. mixed oligoastrocytoma) showed independent prognostic significance (p=0.009, Exp(B)=7.370; p=0.003, Exp(B)=3.422 and p=0.022, Exp(B)= 0.351). CA IX positivity, older age and astrocytic component predicted poorer outcome.

2. Reference 34. Reference 34 is the first report where M75 antibody (CA IX antibody) has been described.

Discretionary Revisions

1. More treatment data. Our study is a multicenter study. For that reason, it is not possible for us to have the treatment data from all the patients, although the clinical practice is quite congruent in Finland. However, because the treatment of oligodendrogliomas is usually considered case by case and it differs from the treatment of astrocytomas in many ways, we feel that we cannot provide more specific treatment data based on just those patients operated in our own hospital, and it would be inaccurate.