Author's response to reviews

**Title:** MRP3: A molecular target for human high-grade glioma immunotherapy

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**Author's response to reviews:** see over
Dear Dr. Norton:

Re: MS 2112818094357657 - MRP3: A molecular target for human high-grade glioma immunotherapy.

Response to reviewer comments

In reply to the May 17 decision letter from Miss Angelina Ilievska on behalf of Dr. Ingunn Holen, Senior Scientific Editor, BMC-series journals, we would like to submit the enclosed revised version of the above-referenced manuscript. In response to the comments of reviewers, we have increased the number of glioblastoma multiforme (GBM) patient samples, retrieved all patients’ up-to-date medical records, and re-analyzed them for this study, and we have taken more factors into account, such as KPS, extent of resection, and post-operative treatment. Because of the difference of histology and small number of anaplastic astrocytoma (AA) cases, we reported only the highest-grade brain tumor, GBM, for this study. Therefore, the new title will be “MRP3: A molecular target for human glioblastoma multiforme immunotherapy.”

The authors have carefully read the comments of the two reviewers and revised the manuscript accordingly. The comments of each reviewer and our point-by-point responses are listed below.

Title: MRP3: A molecular target for human high-grade glioma immunotherapy

Reviewer #1’s report

Reviewer: Steven De Vleeschouwer

The authors are to be gratulated for the interesting research they performed. This work reflects a very relevant field of research for innovative anti-glioma therapies. The methodology is sound and the possible future applications promising. Some smaller critical reflections, however, can be made.

Major Compulsory Revisions

1. At several occasions throughout the manuscript there is a confounding use of and referral to either an absolute absence of expression of MRP3 in normal tissues and a relative overexpression of MRP3 in glioma tissues. The fact that ‘3 and 10-fold expression’ is used, only justifies the use of a relative notion of expression. In e.g. the second paragraph of the discussion (first sentence) or e.g. the last sentence of the first paragraph on p.16), the notion of ‘no expression of MRP3 in normal tissue’ is erroneously being used in a rather absolute term, internally conflicting with the notion of ratio’s to support the overexpression of MRP3 in glioma tissue. In the manuscript, the authors should consistently use the more relative notions of expression.

Response: We thank Reviewer #1 for this suggestion, and we have made corrections accordingly on pages 5, 16, and 21.
2. First paragraph of the discussion: talking about IDEAL antigens for immunotherapy, the authors should rephrase the prerequisites. As it is written in the current manuscript, it seems as if a ‘weakened version’ of the ideal characteristics is being used to maximally fit with the data of MRP3. The ideal antigen should a) be expressed uniformly and exclusively in tumor tissue, b) not (at all) in normal tissue, c) indeed being expressed in an accessible fashion in the tumor cell or environment at a sufficiently high density and d) being crucial for tumor (cell) survival. By rephrasing it this way, it becomes clear that MRP3 is indeed promising, but not yet the magical bullet.

Response: We thank Reviewer #1 for the suggestion to rephrase the prerequisites. However, we did not present a “weakened” version of targeting, but the one that is practical—and anti-MRP3 meets those criteria. Targeted antigens are rarely if ever expressed uniformly, and may or may not be crucial for tumor cell survival—some just mark the neoplastic process. We never made any claims that anti-MRP3 MAbs are the magic bullets—see Introduction on page 4 about our well-developed sense of tumor heterogeneity, serial antigen expression and targeting. Nevertheless, we have revised the first paragraph in Discussion as follows. “In an ideal situation, target antigens for passive immunotherapy should be (1) tumor specific, i.e., expressed in tumor and not normal tissue, (2) accessible (cell surface or matrix) at a density sufficient for targeting, and significant cell kill. The criterion of absolute tumor specificity is rarely met, and current approaches often must target tumor-associated antigens (ratio of neoplastic tissue to normal tissue expression acceptable for lack of bystander effect).”

3. P.22, last sentence. The optimal route for administration of mAb’s to tumor in the CNS is mentioned to be the local delivery in (or around) the resection cavity, eventually using convection enhanced delivery. A rather old reference (48) is being used to support this notion, but it should be made clear to the readership that especially these focal deliveries have important limitations: passive microdiffusion only happens to a very poor amount given the gliosis reaction in the wall of the surgical cavity and convection enhanced delivery should take into account modern calculations of isodose delivery distributions to really cover the relevant areas.

Response: We thank Reviewer #1 for pointing out that the limitations should be mentioned. We have revised the paragraph as follows. “…the optimal route for the administration of MAAb-based therapeutic agents for tumors localized within the CNS is through surgically created resection cavities or saturation of an entire hemisphere by intracranial microdiffusion, called convection-enhanced delivery, to the brain tumor [48], which allows direct parenchymal infusion of therapeutics, bypassing the blood-brain barrier. Only trace amounts of therapeutics distribute systemically, and the possibility of life-threatening side effects, such as lung edema, would be minimal. However, these focal deliveries have limitations: Passive microdiffusion happens to only a very poor extent, given the gliosis reaction in the wall of the surgical cavity, and convection-enhanced delivery must take into account modern calculations for isodose delivery distributions to cover the relevant areas [49].”

Minor Essential Revisions
1. On page 19, second paragraph under ‘Immunohistochemical analysis’, reference to figure 4 is used three times instead of figure 3: this should be corrected.

Response: We have made the corrections accordingly.

Discretionary Revisions
1. It is not clear why the authors use the age cut-off value of 45 years in the survival analysis: median age in this patient population is between 55 and 60 making ‘45 years’ a bit arbitrary as a cut-off value. If they built on specific publications, it might be good to acknowledge them.

Response: We have used the age cut-off of 45 years in the survival analysis, building upon operation
judgement and our previous analysis published in Clinical Cancer Research (Kuan et al, 2006 ref #9), which we cite on page 15, under Statistical Analyses, in the Methods section.

Reviewer #2’s report
Reviewer: Hilko Ardon

Title: MRP3: A molecular target for human high-grade glioma immunotherapy

Description: This is a well written and easy to read manuscript on MRP3 as potential target for human high-grade glioma (HGG) immunotherapy. The authors describe that MRP3 is overexpressed at both mRNA and protein levels in HGG, and that this overexpression is correlated with higher risk of death. From these data Kuan et al. conclude that the tumor-associated antigen MRP3 has a potential use as a prognostic predictor for malignant gliomas and might be useful in immunotherapeutic strategies for treating malignant glioma patients.

Comments

Major Compulsory Revisions:

1. Relative MRP3 mRNA expression levels, IHC, and age were considered as possible predictors of survival and survival analyses were made based on these three factors. As one could expect age was a strong predictor of survival, as were high levels of mRNA expression. However, it is unclear from the reported data if other known prognostic factors, such as RPA classification, Karnofsky Performance Score and grade of resection, were taken into account. Also, it is unclear if all patients received the same postdiagnostic treatment, since differences in survival might be due to differences in treatment. Therefore, it is mandatory to see the demographic and treatment details of the different patients before conclusions can be drawn on the prognostic value of MRP3 mRNA expression.

Response: We thank Reviewer #2 for this important suggestion. We have retrieved all patients’ up-to-date medical records and re-analyzed them for this study, and we have taken more factors into account, such as KPS, extent of resection, and post-operative treatment. As noted in the Methods section on page 14, these factors are now included in our revised Table 1, “Patient Characteristics,” to show the demographic and treatment details.

2. Based on the legend of Fig.1 it can be concluded that all the results for normal brain tissue (MRP3 mRNA levels) come from 1 sample (NWB (normal whole brain) n = 1) (?). A bigger sample size would strengthen the claims of the authors.

Response: We thank the reviewer for the suggestion. We have repeated the study in four independent real-time PCR experiments, including six normal whole-brain samples as the baseline standard.

Minor Essential Revisions

1. It would be interesting to see MRP3 IHC results for normal brain, since expression is expected to be absent.

Response: We indeed have done the normal CNS histology with anti-MRP3 rabbit serum 1708 or Mabs such as 16A11, and no IHC staining was observed.

2. The legend of Fig. 2 should be changed; quantitative FACS analysis should point to Fig. 2 (C) and (D).

Response: We have revised the legend of Fig. 2 according to the reviewer’s suggestion.
3. It is unclear whether the Results of the Statistical analyses are based on uni or multivariate models (as described in Methods). This should be clarified.

Response: We have done the statistical analyses based on univariate and multivariate models, as stated in the Abstract, and in response to Reviewer #2, we have rewritten the Methods and Results sections.

4. The Conclusions section of the Abstract should be rewritten (incorrect sentence).

Response: We have corrected the conclusion of the Abstract as follows. “Human GBMs overexpress MRP3 at both mRNA and protein levels, and elevated MRP3 mRNA levels in GBM biopsy samples correlated with a higher risk of death. These data suggest that the tumor-associated antigen MRP3 has potential use for prognosis and as a target for malignant glioma immunotherapy.”

Discretionary Revisions

1. The first paragraph of the Discussion starts with a description of the ideal antigens for immunotherapy. Besides the three characteristics already mentioned, the immunogenic potential of an antigen (especially in the case of active immunotherapy) is also a key-feature of an ideal antigen. Moreover, tumor-associated antigens (TAAs) for immunotherapeutical use should be present on the tumor cells that are responsible for recurrence of the tumor (since HGG are very heterogeneous it can be expected that not all tumor cells will express the same TAAs).

Response: We thank Reviewer #2 for the suggestion, and we have revised the first paragraph as follows. “In an ideal situation, target antigens for passive immunotherapy should be (1) tumor specific, i.e., expressed in tumor and not normal tissue, (2) accessible (cell surface or matrix) at a density sufficient for targeting, and significant cell kill. The criterion of absolute tumor specificity is rarely met, and current approaches often must target tumor-associated antigens (ratio of neoplastic tissue to normal tissue expression acceptable for lack of bystander effect).” This rephrased paragraph stresses passive immunotherapy that we presented in this study. Immunogenic potential is not a key here. The question of heterogeneity is clearly addressed on page 4 in the Introduction. We are well aware of serial changes in antigenic phenotype. We believe that it is not a necessity for TAAs to be responsible for tumor recurrence - all that is required is demonstration of expression.

2. Table 2 does not seem to add much to the manuscript and a description in the text would be sufficient.

Response: We thank Reviewer #2 for the suggestion. However, we feel that the reader might appreciate the additional information showing that there is no cross-reactivity among the MRPs by the MAbs we raised.

3. In the first alinea of the Background it is stated that ‘mean postdiagnostic survival for HGGs remains less than one year’. More recent studies point to a longer median overall survival: 14.6 months since Temozolomid (TMZ) was introduced in the postoperative treatment (surgery – radiochemotherapy (TMZ) – maintenance TMZ chemotherapy) (Stupp et al., N Eng J Med 2005).

Response: We have revised the sentences in the Background section as suggested, as follows. “Although a recent study showed meaningful survival benefit associated with chemotherapy using a temozolomide-based chemoradiation approach [1], the median progression-free survival among patients treated with this regimen was only 7.9 months, and the overall survival was only 14.6 months [1]. Thus, an effective treatment for GBM patients is still a critical need.”
Again, we thank Reviewers 1 and 2 for these critical comments. We believe that the revisions we have made in response to these comments have significantly improved our manuscript.

Sincerely yours,

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