Author’s response to reviews

Title: TLR9 expression in glioma tissues correlated to glioma progression and the prognosis of GBM patients

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Author’s response to reviews: see over
Dear Editors,

The enclosed is my revised manuscript entitled ‘**TLR9 expression in glioma tissues correlated to glioma progression and the prognosis of GBM patients**’. The manuscript was revised according to the reviewer’s comments. All changes are highlighted with yellow in the manuscript, point-by-point response to reviewer’s comment is appended below.

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Thank you very much for your attention to our paper.  
Sincerely yours,
Chao et al. address interesting question regarding the relation between TLR9 expression, glioma progression and prognosis. Previous studies in mouse tumor-models have demonstrated that administration of TLR9 agonists can have positive effects on induce anti-tumor responses. Some controversies exist in literature as to whether TLR9 activation is beneficial in all circumstances and to which cells in glioma tissues express TLR9.

Major points:

A significant part of the data (tables and figures) presented by Chao et al. are not novel but rather confirm data present in literature:

1) TLR9 is expressed on glioma tissue samples and glioma cell lines on RNA level (Meng et al., Andalussi et al.) and on protein level: Meng et al.)

Our reply: Expression of TLR9 has been reported at several cancer cells including lung[1], gastric cancer[2] and breast cancer cells[3], which suggested that TLR9 mediated invasion may be a general feature of cancer cells. However, whether or not the expression of TLR9 is a common event in glioma is unknown. There are controversy results for the expression of TLR9 in glioma cells, Grauer et al[4] showed that TLR9 are essentially absent at GL261 cells, While Andaloussi et al[5] found a strong expression of TLR9 in GL261 cells with RT-PCR and FCM analysis. This difference was most likely not caused by the contamination of genomic DNA at RT-PCR analysis, since Andaloussi et al [5] further confirmed their RT-PCR result with FCM. We preferred Grauer’s data because their result is consistent with the function of TLR9 signal pathway. As a receptor for CpG ODN, TLR9 play a key role in tissue repair and cancer progression, it was demonstrated that CpGs stimulated the invasion of TLR9+ cancer cells, but not TLR9- cancer cells[6]. Grauer et al [4] showed that GL261 is TLR-, which indicated that CpG ODN may not affect the invasion of GL261 cells, this was supported by widely accepted results which showed that CpG ODN induced apoptosis of GL261 in vitro and cured GL261 glioma animal model in vivo.

Meng et al [7] and Andaloussi et al [5] found that U87 and U251 express high level of TLR9 mRNA, however no data about the expression of TLR9 protein in these two cell lines was reported. To avoid the controversy data like GL261, we investigated the expression of TLR9 in U87 and U251 cells at both the mRNA level(RT-PCR) and Protein level(immunofluoresence), our result for the first time showed that TLR9 staining located intracellularly and could be seen throughout the cytoplasm in U87 and U251 glioma cells.

(page 15 and page 16)
2) TLR9 is expressed on glioma tissue samples and glioma cell lines on protein level (Meng et al.)

Our reply: The relationship between the expression level of TLR9 and tumor grade of glioma is unknown, to investigate this, large amount of clinical samples are required. Meng et al [7] checked the expression of TLR9 in 37 frozen glioma samples, and they found no relationship between the TLR9 expression and survival, however, there are several factors may affect their results in their study: 1.some of the their samples were frozen for a very long time since 1990. 2.their samples were frozen under -180°C, the frozen and thawing process may damage the cells and thus affect the result of immunohistochemistry analysis. 3.for the QPCR experiment, they set a QPCR threshold (0.025) to divide the patients into low TLR9 group and high TLR9 group, however, this threshold is not a widely accepted criteria and may affect result of the analysis. 4. Only quantitative RT-PCR data was used and there is no quantitative protein level data. The non-neoplastic brain tissues and necrotic tissue, which have low expression of TLR9, may be included in the tissues for the extraction of mRNA from the specimen, and thus may interfere with the result. Because of these reasons, their results require confirmation with larger specimen numbers. So we investigated the expression of TLR9 protein in large collection of glioma samples with tissue array. Different to their result, Our study demonstrated that TLR9 expression increases according to the histopathological grade of glioma, and the TLR9 expression level is related to the PFS of GBM patients. (Page 16)

3) Increased in-vitro invasiveness of glioma cell line after CpG stimulation (in fact, Merrell et al. demonstrate that this is induced by secretion of matrix-metalloproteinase-13).

Our reply: Merrell et al [6] showed that treatment of TLR9 expressing breast and brain cancer cells (U373 and D54MG) with CpG ODN stimulates their invasion. In our research, a different glioma cell line (U87 cells) were used to ensure whether or not TLR9 is responsible for the invasive or proliferation of glioma cells induced by CpG ODN. U87 cells were treated with the CpG with or without chloroquine, an inhibitor of TLR9 signaling. Our results suggest that TLR9 signal pathway was responsible for the CpG induced invasion of U87 cells. Merrell et al [6] indicated that CpG ODN induced the invasion of glioma cells via MMP3, however, Other mechanism or other MMP molecule may also involved, The invasion ability of glioma cells is very complicated and the exact underlying mechanism required further researches and is out of the focus of this study. (Page 17 and Page 18)

The aim of our research is to investigate whether or not the TLR9 expression is related to the development and prognosis of glioma, the TLR9 expression status in cell lines and in large amount of samples is the basis for our research. As explained above, all these data are necessary and most of them except the data about mRNA expression in U87 and U251 cells are novel. The underlying mechanism of TLR9 induced invasion of glioma cells is out of the focus of our paper.
Furthermore, TLR9 mRNA expression is rather difficult to analyze as it is encoded by a single exon gene. Without DNase treatment on the RNA samples, inclusion RT+ and RT- samples, and semi-quantitative PCR it is difficult to draw unequivocal conclusions regarding the presence or absence of physiologically significant amounts of TLR9 mRNA. Also regarding the TLR9 staining it remains unclear how specific this staining really is as a true positive control and a negative control (isotype matched primary control Ab) are missing. Finally, also a number of the functional experiments are poorly controlled.

**Our reply:** In this research, we did not think the data about TLR mRNA expression was false-positive results caused by genome DNA contamination, since the expression of TLR9 in U87 and U251 cells were further confirmed by immunofluorescence analysis, and the expression of TLR9 in glioma tissues were further confirmed by immunohistochemistry method. (Page 7)

For the TLR9 immunohistochemistry staining, the anti-TLR9 antibody (Img-305A, clone 26C593.2, Imgenex, San Diego, USA) was widely used by other researchers. In our research, during immunohistochemistry staining, primary antibody control was replaced by normal mouse serum, which was widely used as negative control in immunoassay. Most importantly, we demonstrated that there is no TLR9 staining in normal brain tissues, which indicated no false positive staining exist in our study. It was demonstrated that kidney tubule but not the glomerus have the expression of TLR9[8], so we used kidney tubules as positive control for TLR9 staining in this study. (Page 12)

The claim of Chao et al. that high TLR9 expression (in 128 grade IV gliomas) correlates with poor prognosis is in itself interesting. Others did not find such a correlation (Meng et al.). Unfortunately, patient selection, patient-characterization, parameter-definition and method to address this point are completely insufficient. This opinion will be highlighted by giving some examples:

**Our reply:** It is not surprise that we got different results with Meng et al [7], as indicated above, there are several factors may affect their results: 1. some of the their samples were frozen for a very long time since 1990. 2. For the QPCR experiment, they set a QPCR threshold (0.025) to divide the patients into low TLR9 group and high TLR9 group, however, this threshold is not a widely accepted criteria and may affect result of the analysis. 3. Only quantitative RT-PCR data was used and there is no quantitative protein level data. The non-neoplastic brain tissues and necrotic tissue, which have low expression of TLR9, may be included in the tissues for the extraction of mRNA from the specimen, and thus may interfere with the result. (Page 16)

We investigated the TLR9 expression in large amount of glioma samples with tissue array analysis. The aim of our research was to investigate whether or not the TLR9 expression is related to the development and prognosis of glioma. We found that Human glioma cells could express functional active TLR9 which can response to CpG ODN, the TLR9 signal pathway may play a role in the glioma development and the expression of TLR9 is related to PFS of glioma patients. The patient selection, patient-characterization, parameter-definition and methods used were carefully described in the manuscript and are sufficient to achieve our conclusion.
1) Chao et al. analyse not all 128 patients in their study but select 69 patients that are recurrent or dead before July 2009. This selection criterion may introduce a strong selection-bias.

**Our reply:** For the survival analysis, to avoid the bias caused by different treatment strategy, in addition to the screening criteria indicated in the methods part, patients must received similar treatments strategy: surgical resection was performed by neurosurgeons who used similar operational techniques and principles. Every patient in the study received the same radiotherapy regimen. All Patients received chemotherapy after radiotherapy. The criteria ensured that the patient group was uniform, which strengthens the analysis. We stopped to collect data at July 2009, the observation for each patient will end once the patient is dead or have recurrent. 69 GBM patients fulfill these criteria and were used for the survival analysis. There are no patients survived more than 24 months. (Page 6)

2) Table 3 in fact suggests that selection-bias may have occurred because clinical parameters that have been previously shown to strongly influence prognosis in GBM patients are not correlated to PFS in this study (KPS, Extent of resection; table 3).

**Our reply:** For the survival analysis, our research include same group of patients that was used in one previous published study[9], which showed that the resection extent and KPS were not related to the survival, and these data were further supported by other researcher’s results, based on the published literature, it remains unclear whether the extent of surgical resection correlates with survival[10-12]. Even for KPS, there are many studies found that it is not associated with survival of GBM patients[13-15]. So the prognosis value of clinical parameters such as extent of resection and KPS should not be considered as indicators for bias of patient selection. (Page 17)

3) One of the clinical parameters the authors use is DFS: disease free survival?. On page 12 the authors conclude that extent of resection were not associated with DFS in GBM?. This is curious, if you have complete resection of the GBM you have a DFS > 0 days. If you have a partial resection one must conclude that the DFS is 0 days.

**Our reply:** Thanks very much for the carefully review, we are very sorry, we made a mistake for the editing work, the word ‘DFS’ should be ‘PFS’ in the manuscript , we have corrected all these editing mistakes.

In conclusion, our findings warrant caution in the directly injection of TLR9 into glioma tissues for the glioma immunotherapy, this conclusion may be contradictory to some researcher’s work[4, 16, 17]. Although there is contradictory results for the expression of TLR9 in the GL261 cells between Grauer’s result[4] and Meng’s result[7], both of them confirmed that the prolonged survival seen in their study was not due to the expression of TLR9 expression in glioma cells, but...
rather due to increased apoptosis of GL261 cells in vivo and enhanced modulation of the local CNS immune response. TLR9 expression in non-tumoral cells or microglia activation may play an important role in the CpG induced anti-cancer immunotherapy, however, the inflammatory response observed following CpG treatment do not always prevent growth of solid tumor masses, in one CpG treated glioma animal model[18], intratumoral application of CpG proved ineffective. In addition to this, Deng et al[19] showed that intracerebral injection of ODN containing CpG motif induce meningitis in mice and rat. In a phase II trial evaluating the efficacy of CpG in 34 recurrent GBM patients[20], patients usually experienced transient neurological worsening or fatigue after intratumoral application of CpG. In 3 patients, seizure occurred just after administration of CpG. Our research indicated that locally administered CpG ODN may increase the invasion of glioma cells. Based on these data, carefully safety evaluation in animal model and in large amount of glioma patients should be performed before CpG can be routinely intratumoral administered in clinic. (Page 18 and 19)

References:


