Higher expression of High-mobility Group AT Hook Protein 2 (HMGA2) in the border zone compared to the center zone of glioblastoma and a tendency that high HMGA2 expression in the border zone may have a poor impact on survival

Amin I. Nohman (amin.nohman@med.uni-heidelberg.de)
Department of Neurosurgery, University Hospital Heidelberg

Frank P. Schwarm
Department of Neurosurgery, University Hospital Gießen

Marco Stein
Department of Neurosurgery, University Hospital Gießen

Anne Schänzer
Department of Neuropathology, University Hospital Gießen

Christian Koch
Department of Anesthesiology and Intensive Care Medicine, University Hospital Gießen

Eberhard Uhl
Department of Neurosurgery, University Hospital Gießen

Malgorzata Kolodziej
Department of Neurosurgery, University Hospital Gießen

Research Article

Keywords: Glioblastoma, HMGA2, border zone, infiltrative zone, biomarker, invasiveness

Posted Date: April 8th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1526416/v1

Abstract

Introduction: High-mobility group AT-hook protein 2 (HMGA2) is a gene regulatory protein that correlates with malignancy, metastatic potential, and poor prognosis. It has been shown that HMGA2 is overexpressed in various tumors such as esophageal squamous cell cancer, lung cancer and pancreatic cancer. The invasive character and highly aggressive structure of glioblastoma led us to invest the HMGA2 expression in the border zone of the tumor more closely. We compared the HMGA2 expression in glioblastoma and normal brain tissue. Also, we analyzed and compared the HMGA2 expression in the border zone and center zone of the tumor. HMGA2 expression was additionally correlated with clinical parameters such as sex, age, MGMT methylation status, progression-free survival, and overall survival.

Methods: Samples from 23 patients with glioblastoma WHO grade 4 were analyzed for HMGA2 expression using quantitative real-time polymerase chain reaction (qPCR) and immunohistochemistry (IHC). Areas from the tumor center and border were analyzed separately. Two normal brain tissue specimens served as controls. Results were compared with molecular results and clinical parameter.

Results: Both, real-time PCR and immunohistochemistry displayed a significantly higher HMGA2 expression in glioblastoma compared to normal brain tissue (qPCR p=0.013, IHC p=0.04). Moreover, immunohistochemistry showed a significantly higher HMGA2 expression in the border zone of the tumor than in tumor center zone (p=0.012). Survival analysis revealed a tendency to a shorter survival if HMGA2 was highly expressed in the border zone. No significant correlation between HMGA2 expression and survival was obtained.

Conclusion: In summary, the results display an overexpression of HMGA2 in the border zone of glioblastomas and thus thorough surgical resection of the vital and aggressive border cells might be important to inhibit the invasive character of the tumor.

Key Points

Question: Is the expression of HMGA2 higher in the border zone of Glioblastoma compared to the center zone and does it contain a prognostic relevance?

Key conclusion: With these results we show that the tumor border in glioblastoma might carry key factors and proteins that support invasiveness and bad prognosis and thus the specific therapy of the border zone might be relevant for future therapy strategies.

Introduction

Glioblastoma is the most frequent and most malignant brain tumor in adults with an incidence of 4–5 cases per 100.000 per year (1). Despite all the effort of improving the outcome of the patients, median overall survival (approximately 15 month) is still very low (2). Currently, the standard therapy consists of three main methods. Most of the diagnosed glioblastoma patients undergo surgery before receiving

concomitant radio- and chemotherapy and adjuvant chemotherapy (3). Standard of care in regards of chemotherapy is temozolomide. The combination of temozolomide with radiotherapy prolongs the survival significantly (4). Especially glioblastoma with a positive methylation status (MGMT) showed good results when treated with temozolomide (5). Molecular constitution, like the MGMT methylation status or isocitrate dehydrogenase (IDH) mutations, seems to regulate the responsiveness and treatability of glioblastoma. In the updated WHO classification of 2016, molecular markers such as MGMT or IDH are decisive and have a great impact on the classification, prognostic value and therapy of glioma (6). An even greater focus on molecular features in gliomas is also emerging in the updated WHO classification announced for 2021 (7). More molecular markers, such as HMGA2, might be following and become more important in the future. Learning more about these molecular markers might improve the efficiency and outcome of treatment (8).

High-mobility group (HMG) proteins are DNA binding factors with transcriptional and cell activating functions. HMGA binds to adenine and thymine (AT-hook) rich DNA in the minor groove of the double helix (9). It bends, straightens, unwinds and induces looping in linear DNA molecules (10) and thereby activates protein synthesis and cell activity (11). By changing the cell formation and inducing an epithelial-mesenchymal-transition (EMT), HMGA2 carries a crucial role in malignant degeneration and cell migration (12). Moreover, oncogenic pathways like the RTK/RAS/PI3K pathway are upregulated, while tumor suppressing factors, such as the microRNA family let-7 pathway, are downregulated in HMGA2 positive tumors (13). HMGA2, which is genetically located in chromosome 12q14, is physiologically expressed in embryonal cell development (14). In differentiated adult cells, HMGA2 is usually not detectable, unless these cells undergo a malignant transformation and thus HMGA2 marks and distinguishes tumor cells from normal healthy somatic cells (15).

Studies have shown that various malignant tumors, such as colorectal (16), lung (17), neuronal (18) and other types of cancer, show an HMGA2 overexpression. In addition, high HMGA2 expression was also linked with distant metastasis (19), increasing invasiveness (20), larger sized tumors (21) and poor prognosis (22). HMGA2 appears to promote the self-renewal capacity of glioblastoma and hence boosts stemness and tumorigenicity (23). Adding to these findings, studies demonstrated that HMGA2 depleted glioblastoma cells show less migration and invasion (24, 21). Schwarm et al. showed that glioblastoma present an overexpression of HMGA2 and that this overexpression of HMGA2 results in a tendency of shorter progression-free survival (PFS) and overall survival (OS) (25).

Since no previous study focused on the distribution of HMGA2 and its relevance on glioblastoma, we explicitly compared tissue from the tumor border and central zone. The idea was that HMGA2 with its oncogenic characteristics is highly expressed in the vital tumor border and that such molecular markers shift from the hypoxic and necrotic tumor center to the border zone. Also, we wanted to examine if high HMGA2 expression in the border zone could be a marker for bad prognosis in glioblastoma.

Material And Methods

Patients and tumor samples

Between 2016 and 2018, twenty-three adult patients were included. All patients were newly diagnosed with glioblastoma and thus did not undergo earlier treatment. The included patients received gross total resection where we collected tissue samples from the tumor border zone and the center zone. Further they were treated according the Stupp protocol (4). See detailed patient information and clinical data in table 1. Also, two samples of normal brain tissue, which we kindly received from our colleagues of the institute of pathology in Salzburg were tested and compared to the tumor samples.

Table 1 Patients baseline information

Baseline characteristics	N (%)
Total cases	23 (100)
Age at diagnosis	57.8±8.4 years
Gender	
• Male	16 (69.6)
• Female	7 (30.4)
MGMT Status	
• Methylated	10 (43.4)
• Unmethylated	13 (56.5)
IDH Status	
• Mutation	0 (0)
• Wildtype	23 (100)
Survival Data	
• Overall survival	14.3±8.7 month
• Progression free survival	7.26±6.7 month

The samples were preserved in either liquid nitrogen for qPCR analysis or formalin fixed for standard pathological diagnosis and for immunohistochemistry. The definitive diagnosis was made by experienced neuropathologists. Finally, HMGA2 expression was correlated with clinical parameters such as sex, age, and survival data. In addition, we compared and correlated the HMGA2 expression in the border zone to known markers like MGMT status. In our collective there were 10 methylated and 13 unmethylated glioblastoma, whereas all 23 patients bared IDH wildtype glioblastoma.

Ethical standards

The work was approved by the ethical committee of the University of Giessen (AZ07/09) and written informed consent was obtained from the patient. The study was conducted in accordance with the principles of the Declaration of Helsinki.

qPCR

To isolate RNA from the intraoperatively obtained brain tissue, we used the RNeasy Mini Kit® from Qiagen (Qiagenã, Hilden, Germany) and followed the steps according to the protocol. The obtained RNA was photometrically measured with the peqLab NanoDrop® (peqLABã, Erlangen, Germany) 1000 Spectrophotometer to use the right dosage for the translation of RNA to complementary DNA (cDNA). We used 1µg of the RNA and performed the cDNA synthesis using Qiagen's QuantiTect RT Kit®. For the following qPCR we pipetted cDNA of each sample with MasterMix®, water and lastly added different human primers. We used the HMGA2 primer (Hs00171569_m1). For the endogenous controls we used the actin-β- (Hs99999903), IPO8- (Hs00914040) and TBP (Hs00427620) primer. The setting of the qPCR cycler was set according to the manual (Mastercycler Gradient Thermal Cycler®, Eppendorf Hamburg, Germany). Moreover, we controlled and compared the results with standardized control curves and gene expression to exclude pipetting or other errors. To ensure correct work, we limited the standard deviation of each sample to 0.25. We used the cycle threshold (Ct) values to calculate the gene expression. First, we determined the mean Ct value of all the endogenous controls. This mean Ct value was then subtracted from the HMGA2 Ct value resulting in a ΔCt. To finalize the calculation and get the expression level, we used $2^{-\Delta Ct}$ to adjust according to the exponential nature of qPCR.

Immunohistochemistry

From formalin-fixed, paraffin embedded (FFPE) tissue samples 3 micrometer sections were used for immunohistochemistry. In brief, deparaffinized sections were incubated with HMGA2 antibody (ab52039; Abcam, Cambridge, UK; 1:50) for 60 minutes and visualized with Zytomed's DAB Substrate Kit High Contrast®. Tissue samples from breast cancer samples served as positive controls.

To evaluate the HMGA2 expression, we used a Leica DMLB microscope equipped with a digital camera. The HMGA2 positive nuclei of five different locations of each IHC stain were manually counted at 40x magnification. In addition to the number of colored cells, the intensity of staining was scored and graded into weak, medium, and strong. Semi-quantitatively, we evaluated HMGA2 expression by accounting the intensity of staining with the number of cells colored. The investigator (A.N) was blinded to the specimens.

Statistical analysis

For statistical evaluation we used the Mann-Whitney-U test to compare the different HMGA2 expression rates in the border zone and in the central-zone specimens of the tumor. These results were compared and analyzed with the expression rate of the normal brain specimens. Also, we correlated the HMGA2 expression rate with clinical parameters such as age, sex and MGMT methylation using the Mann-

Whitney-U test. Finally, we used Kaplan-Meier curves to show the correlation between HMGA2 expression and OS and PFS. To compare OS and PFS, we separated the cohort into a high HMGA2 expression group and into a low HMGA2 expression group and used the median HMGA2 expression in the border zone of the tumor as a cut off. All patients with a HMGA2 border zone expression above that median value were assigned to the high HMGA2 expression group. All the other patients with lower HMGA2 expression than the median value in the border zone, were grouped as low HMGA2 expression. This separation makes it possible to look more closely at the effect of above-average HMGA2 expression in the border zone since we believe that the higher the HMGA2 level in the border zone, the worse is the spreading characteristics and prognosis. p-values <0.05 were considered significant in all tests.

Results

Descriptive analysis

Median age of the included patients was 57±8 years. According to the literature, men were slightly more likely to develop glioblastoma, so that the ratio between men and women was 2.3:1. MGMT promoter methylation was detected in 10 of 23 patients. None of the included patients showed an IDH mutation. For survival analysis, we examined overall and progression-free survival. In our patient population, a median OS of 13-month (interquartile range (IQR) = 14 month) and a median PFS of 6-month (IQR = 8 month) were recorded.

HMGA2 expression in glioblastoma

Comparing the HMGA2 expression by performing qPCR and IHC in tumor cells and normal brain tissue specimens, showed significantly higher expression of HMGA2 in the tumor cells (qPCR p = 0.013; IHC p = 0.04). HMGA2 expression in the border zone as well as in the center zone of glioblastoma was significantly higher than in normal brain tissue (Fig. 1).

Test results comparing border and central parts of the tumor showed higher HMGA2 expression in the border zone of the tumor only at protein level using IHC (p = 0.012; Fig. 2). qPCR did not show significant differences (p = 0.277).

Correlating HMGA2 expression with age and sex showed no statistical difference (correlation with age qPCR = 0.905; IHC = 0.73; correlation with sex qPCR = 0.974; IHC = 0.82). In addition, no correlation of HMGA2 expression of MGMT methylation status could be observed. Furthermore, the results also failed to show any effect of the MGMT on the HMGA2 expression in the border zone of glioblastoma. Thus, the HMGA2 expression in the border zone did not vary significantly in MGMT methylated or unmethylated glioblastoma samples (qPCR = 0.68; IHC = 1). In our study, the MGMT methylation appears to carry a minor role in HMGA2 expression.

High HMGA2 expression in the border zone could have an impact on survival

Overall, our survival data indicate that a high HMGA2 expression in the border zone could have a negative impact on survival. qPCR results suggest that patients with high HMGA2 expression in the border zone, were more likely to have shorter OS (p = 0.291) and shorter PFS (p = 0.231; Fig. 3). Questionably, the IHC results were not as clear (OS p = 0,684; PFS p = 0.687). The median OS for patients with high HMGA2 expression in the border zone was 11 months, whereas patients with comparatively low HMGA2 load in the border zone survived about 16 months. PFS of patients with high HMGA2 expression in the border zone was on average 2.3 times shorter than that of patients with lower HMGA2 expression in the border zone.

We also evaluated the survival in patients with high HMGA2 load in the border zone with or without MGMT methylation (Fig. 4). Interestingly, a high HMGA2 expression at the tumor border in addition to a MGMT negative status, seems to have the tendency of lower PFS (p = 0.182). A combination of a MGMT methylated glioblastoma and high HMGA2 expression in the border zone did not show any tendency. None of the survival tests showed significant differences though.

Discussion

Our hypothesis is that HMGA2 is highly expressed at the border zone. Due to hypoxia protein expression shifts to the invasive front of the tumor and causes infiltration and malignancy (26, 27). For this reason, we were determined to analyze the HMGA2 expression in the border zone. Moreover, we strongly believe that there could be a link between the microstructure and molecular genetics at the tumor border and the fact that most recurrences arise directly adjacent to the resection cavity (28). The importance of the molecular setting of glioblastoma and its influence on treatment recommendation and prognosis estimation is known and with MGMT and IDH two of these markers are stablished diagnostic marker for malignant glioma (5, 29). In previous studies HMGA2 proved to be correlated with bad prognosis and shorter PFS and OS (30). So far, no study examined and compared the HMGA2 expression in different parts of glioblastoma and hence little information is known about the actual expression cluster of HMGA2 in glioblastoma.

Our results confirm that HMGA2 is significantly higher expressed in glioblastoma than in normal brain tissue and HMGA2 is higher expressed in the border zone than in the tumor center. This fact is interesting regarding separation of healthy and normal brain tissue to malign glioblastoma cells. Tumor cells might be distinguished by the overexpression of HMGA2 (31). Single glioblastoma cells at the border zone of glioblastoma might be identified by visualizing these new markers such as HMGA2. This could allow us a better understanding and more information of the spreading and invasive character of glioblastoma and a better labeling of the border zone. Should HMGA2 prove to be a highly relevant and specific marker of the border zone, future approaches of intraoperative visualization by imaging or histopathological methods may show clear differentiation from normal brain tissue. This could improve the surgical outcome. It is still unclear whether HMGA2 is expressed in all glioblastoma types and whether imaging HMGA2 truly represents the invasive nature of the tumor and provides additional beneficial information.

To know the exact difference of HMGA2 expression between glioblastoma tissue und healthy brain tissue, more tests on normal and healthy brain tissue are needed.

It would be worth considering correlating HMGA2 expression with other known hypoxia markers. An interaction between the markers is quite conceivable and could show more insight into the dynamics of the border zone.

Also, high HMGA2 expression in the border zone shows the tendency to correlate with quicker progression and shorter overall survival (Fig. 3 and Fig 4). The overexpression of HMGA2 in the border zone could contribute to the high recurrence rate at the surrounding border zone. The directly adjacent areas appear to be a good host for recurrences, as approximately 80% of recurrences originate locally from the tumor border zone, although it has been demonstrated that at the time of diagnosis some tumor cells have already spread distally in the brain and can even be detected systemically in the bloodstream (32). These results emphasize the importance of the border zone of glioblastoma. Obviously, the sample size and study design are not suitable for accurate survival data and is not conclusive, especially since no significant results could be presented. In addition, except for a few clinical data like age and sex, we did not consider secondary diagnoses or diseases that may also play a role on overall survival. Comparison with large databases that also include molecular genetics would be one way to add a little more power to the survival analysis (33). The cells profile of the tumor border with its gene expression profile such as HMGA2 might also be the reason why gross total resection with an extensive resection of the border zone shows significantly better results (34). A thorough and good resection of the border zone might be crucial and have a bigger impact than the resection of the tumor center. First tests showed that supramarginal resections in non-eloquent areas showed a survival advantage with the same quality of life (35). In the future, tissue samples obtained intraoperatively should possibly also be taken explicitly from the border zone of the tumor and sent to further examination. Should the diagnostics of the border zone tissue show an aggressive genetic profile and overexpression of HMGA2, more aggressive and focused radiotherapy in the adjacent areas might be considered to prevent rapid progression (36). Targeted therapy of the border zone remains difficult because eloquent areas or important structures are often adjacent and surgical removal is limited. Also intensified radiotherapy can also have significant side effects. Before HMGA2 or other molecular markers have a decisive impact on therapy delivery, the relevance of such markers must be clear. The data obtained in this study, recommend further investigation with the goal of developing better therapeutic options for the border zone of glioblastoma.

There is reason to believe that the overexpression of HMGA2 in the border zone is due to a niche at the border zone (37). It is becoming clearer that there seems to be structural differences in the border zone of glioblastoma (38). A high HMGA2 expression in the border zone could be one element of a very lively and active border niche, where glioma stem cells, oligodendrocyte progenitor cells and microglia seem to be overly present (39). A thorough understanding of this microstructure and its effect on glioblastoma and normal brain tissue could be promising.

HMGA2 expression did not vary significantly when stratified by clinical parameters. In our results HMGA2 expression showed no correlation in gender, age or MGMT methylation status. In comparable studies for other tumor entities such as renal carcinoma, the results also did not show any significant differences in sex, age and tumor size (40). Data on HMGA2 expression in glioma, have also failed to proof a positive correlation with gender and age (41). MGMT methylation status did not affect HMGA2 expression in our results. This supports previous data that also showed no relationship between HMGA2 and MGMT methylation status (25). Larger cohorts are needed to explore possible correlation to clinical parameters, although so far there is no indication for such a link.

It could be reasonable if therapeutic strategies were used specially to treat the border zone and thus eliminating as much from the vital tumor border as possible. Targeted inhibition of HMGA2 in other tumor entities and glioblastoma already showed some beneficial results by reducing endothelial-mesenchymal transition and thus decreasing stemness and invasion (42).In terms of surgery, the elimination and resection are mostly limited to the location of the tumor and navigational systems (43). The location cannot be changed, thus gross total resection or even supramaximal resection is dependent on good navigational systems and intraoperative monitoring. Increasing the quality of these tools should be aimed at so that the resection level and depth in the border zone could be elevated (44). Taking advantage of molecular markers in the border zone and combining the visualization of these markers with navigational systems, could increase the resection level in the border zone (45). Intraoperative real-time histology using Rama-Spectroscopy, could provide more clarity on the resection of the border zone in the future (46). Imaging of markers specific for the border zone of the glioma would be a useful next step to improve resection outcome.

Conclusion

This study showed that HMGA2 is overly expressed in glioblastoma. Furthermore, the HMGA2 expression was significantly higher in the border zone of the tumor than in the tumor center and could be possibly linked with the invasive character of glioma. Survival seems to be shorter when HMGA2 is overexpressed in the tumor border, though we could not see significant differences. Future studies should further investigate the role and importance of HMGA2 in glioblastoma and determine the effect of targeted suppression on survival. Over more, intraoperative labeling and information about the border zone of glioblastoma by using biomarkers such as HMGA2, could improve surgical procedure and success.

Abbreviations

HMGA2	High-mobility group AT-hook protein 2
IDH	Isocitrate dehydrogenase
IHC	Immunohistochemistry
MGMT	Methylation of O6-methylguanine-DNA methyltransferase

OS	Overall survival
PFS	Progression free survival
qPCR	Real-time Polymerase chain reaction

Declarations

Acknowledgements: We thank Daniel Neureiter & Hans Ulrich Schlicker (Institute of Pathology, Salzburger Landeskliniken, Salzburg, Austria)

This is a basic experimental study. Molecular tests were done on glioblastoma specimens and then correlated with clinical data. Lastly survival analyzes were completed.

All authors confirm that this is a novel work. All data were obtained independently, and the paper was not published in any other journal prior to this submission.

Ethics approval and written patients' consent were obtained.

Funding: The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing Interests: The authors have no relevant financial or non-financial interests to disclose.

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Amin I. Nohman and F. Schwarm. The first draft of the manuscript was written by Amin I. Nohman. All authors commented on previous versions of the manuscript and then read and approved the final version.

Data availability: The data generated or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval: This study was performed in line with the principles of the Declaration of Helsinki. The work was approved by the ethical committee of the University of Giessen (AZ07/09).

Informed consent was obtained from all individuals participants included in the study.

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Figures

Figure 1

HMGA2 expression in glioblastoma and normal brain tissue. Significantly higher HMGA2 expression were seen at the border zone as well as in the center zone of glioblastoma compared to normal brain tissue (real-time PCR p = 0.013; IHC p = 0.04).

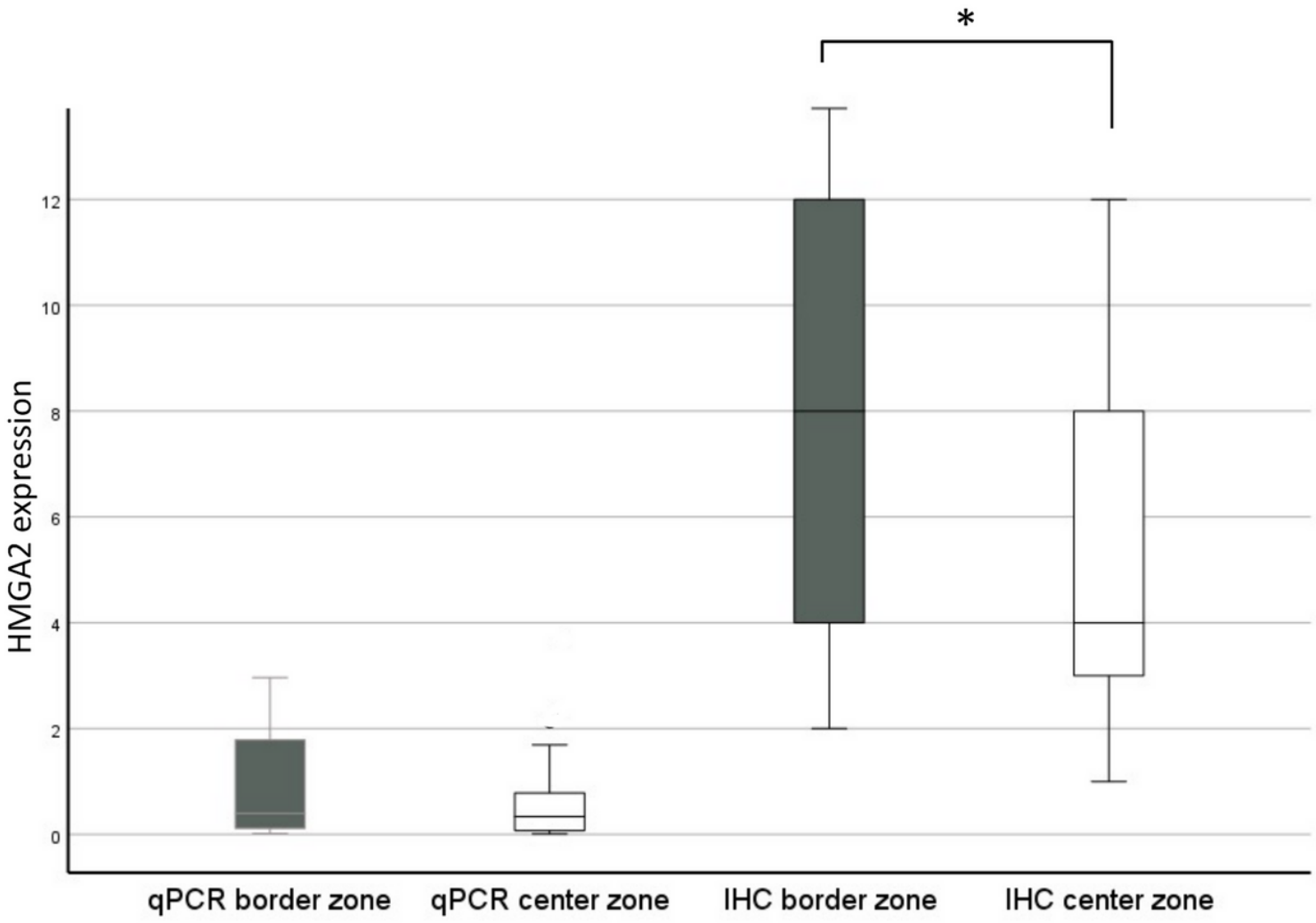

Figure 2

HMGA2 expression analysis using real-time PCR and immunohistochemistry showed higher HMGA2 expression in the border zone compared to the center zone of glioblastoma. A significant difference resulted in the immunohistochemistry (p=0.012).

Figure 3

Comparing survival parameters through OS (A) and PFS (B) in a high and low HMGA2 border zone expression group. The results show a tendency that high HMGA2 expression in the border zone could have a negative impact on survival. These data obtained by real-time PCR (OS p=0.291; PFS p=0.231).

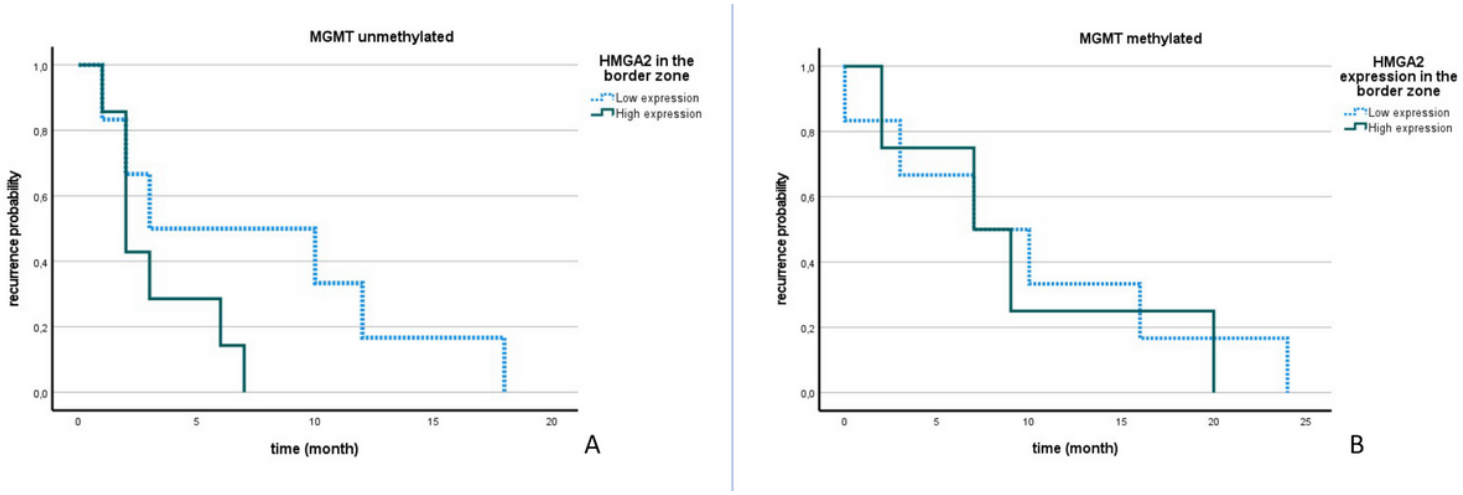

Figure 4

PFS in a high and low border zone HMGA2 expression group stratified for MGMT methylation. The MGMT unmethylated group (A) with high HMGA2 in the border zone showed a possible worse outcome than the patients with high HMGA2 expression in the border zone with a methylated glioblastoma status (B). These data were obtained with real-time PCR (MGMT unmethylated p=0.182).