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SHORT COMMUNICATION

Prognostic significance of MRP5 immunohistochemical expression in glioblastoma

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Abstract
Introduction Glioblastoma multiforme (GBM) is the most frequent malignant primary brain tumor in adults, exhibiting poor survival. The efficacy of chemotherapy is often limited by the development of multidrug resistance by the tumor cells. In the current study, we investigated the prognostic significance of the multidrug resistance protein 5 (MRP5) in patients with GBM.
Materials and methods We retrospectively studied 33 patients with GBM treated with a combination of surgery, postoperative radiotherapy and adjuvant temozolomide chemotherapy. MRP5 protein expression was determined immunohistochemically and correlated with other prognostic factors and survival.
Results The immunohistochemical expression of MRP5 was observed in 0–45% of tumor cells. Patients with MRP5 index >11% exhibited significantly worse survival compared to those with MRP5 index ≤11 (10.5 vs. 18 months, $p = 0.0002$). Patients with Ki-67 index lower than 30% had longer survival (15 vs. 11 months, $p = 0.0084$). Furthermore, patients with a gross total tumor excision had better survival ($p = 0.016$). No significant difference was observed between preoperative Karnofsky performance score, age, gender and survival. In multivariate analysis, MRP5 index and the extent of tumor resection were identified as factors with independent prognostic power.
Conclusion The present results imply that MRP5 index may hold a prognostic role in patients with GBM.

Keywords Glioblastoma · MRP5 · Ki-67 · Prognosis

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Introduction

Glioblastoma multiforme (GBM) is by far the most common type of primary brain tumor occurring in adults. This devastating disease is incurable, and despite aggressive combination treatment, it has poor outcome [1]. Chemoresistance has been reported to be a major obstacle to successful chemotherapeutic treatment for cancer [2]. In patients with GBM, epigenetic silencing via promoter hypermethylation of the DNA repair gene O6-methylguanine-DNA methyltransferase (*MGMT*) has been linked to better response to alkylating agents and longer survival [3]. Apart from *MGMT*, various other genes have been implicated in chemoresistance, among them *MDR1* and *MRPs* [4–10]. The *MDR1* gene encodes the P-glycoprotein (P-gp),

which is an ATP-dependent drug efflux pump. The P-gp efflux pump has been previously showed to be involved in resistance to temozolomide-mediated cytotoxicity [11]. MRPs are members of the ABC superfamily of transmembrane proteins. MRP1, MRP3 and MRP5 have been reported to be expressed more than the other members in gliomas [12–14]. Although MRP1 and MRP3 have been studied in glioma patients, little is known regarding the significance of MRP5 expression [10, 15]. In the present study, we investigated the prognostic significance of MRP5 expression in patients with GBM.

Materials and methods

Patients

We retrospectively studied thirty-three patients who were operated on for primary (de novo) GBM in our institute over a 6-year period (from September 2005 to September 2011). In the pathological specimen, the expression of the MRP5 protein and the Ki-67 index were determined. The extent of resection was determined by comparing MRI scans obtained before surgery with those obtained within a month after surgery. Clinical variables that were analyzed included age, sex, tumor location and preoperative Karnofsky performance status score (KPS). All patients received postoperative standard radiotherapy with concomitant temozolomide followed by temozolomide chemotherapy up to 1 year or until recurrence. Radiotherapy was administered as fractionated focal irradiation at a dose of 2 Gy per fraction given once daily 5 days/week over a period of 6 week up to a total dose of 60 Gy. Follow-up MRIs were performed every 2 months. Tumor recurrence or progression was defined according to the updated assessment criteria for high-grade gliomas [16] and when available on ^{99m}Tc-Tetrofosmin brain SPECT findings [17]. Patients that recurred had only supportive care but no additional surgery or chemotherapy. The study was approved by the Institutional Review Board.

Immunohistochemical staining

Immunohistochemistry was performed on 4-μm sections from formalin-fixed, paraffin-embedded tissue blocks by the avidin biotin technique. For MRP5 staining, the monoclonal antibody anti-MRP5 (NCL-MRP5, Novocastra, dilution 1:50) was employed. Any cytoplasmic staining of tumor cells was considered positive, irrespective of staining intensity. For Ki-67 protein expression, the monoclonal murine antibody MIB-1 (Dako S.A., Glostrup, Denmark) was applied at a dilution of 1:20. All cells with nuclear staining of any intensity were considered positive. In each staining run, tissue sections with omissions of the primary antibody served as negative controls. Our results were expressed as the percentage of positive tumor cells out of the total number of counted cells (approximately 3,000 counted cells). The immunohistochemical expression of MRP5 and Ki-67 proteins was evaluated by two independent pathologists, and any discrepancy in their findings was solved by consensus.

Statistical analysis

Pearson's correlation coefficient was used to correlate between continuous variables. Progression-free survival (PFS) was defined as the time from initial surgery to demonstration of tumor progression on follow-up MRI, or death. Survival time was defined as the time between the date at diagnosis and the date of death for deceased patients or to the last follow-up of the surviving patients. The overall survival time was estimated using Kaplan–Meier methods, and log-rank analysis was performed to compare survival curves between groups. Patients who were still alive at last contact were treated as censored events in the analysis. Multivariate Cox regression analysis of the data was used to analyze possible prognostic factors. The forward stepwise model selection procedure was used (p value of likelihood ratio test <0.05 as inclusion criteria; likelihood ratio test >0.10 as exclusion criteria) to define the final model. The following variables were entered: gender, age at diagnosis, KPS, MRP5 and Ki-67 index and extent of resection. With respect to MRP5 and Ki-67 index, receiving operating characteristics (ROC) curve analysis were performed in order to determine the cutoff value for predicting survival. A two-sided p value <0.05 was considered statistically significant.

Results

Among the 33 patients, there were 20 men and 13 women (median age 60.9, range 54–78). Twenty-four patients had a KPS over 80. In 27 cases, a gross total excision was achieved, whereas in 6 cases, there was a subtotal tumor resection (Table 1). The MRP5 immunohistochemical expression was observed in the cytoplasm of tumor cells and in rare cases in the endothelial cells within the tumor. The MRP5 expression ranged from 0 to 45% (mean 8.8%), and the Ki-67 expression ranged from 1 to 60% (mean 26%). A total of 22 (75%) patients were alive and progression-free at 6 months, and 11 (37%) patients were alive and progression free at 12 months. After a mean follow-up period of 16 months (range 6–36 months), 6 patients were alive.

Table 1 Patients' data

Patients characteristics	*N* (%)	*p* value	
		PFS	OS
Gender			
Male	20 (68.9)	0.78	0.91
Female	9 (31.1)		
Age			
>60	15 (51.7)	0.94	0.96
<60	14 (48.2)		
KPS			
>80	20 (69)	0.1	0.28
<80	9 (31)		
MRP5			
>11%	7 (24.1)	0.0085*	0.0002*
<11%	22 (75.9)		
Ki-67			
<30%	19 (73.1)	0.047*	0.0084*
>30%	7 (26.9)		
Extend of resection			
GTR	23 (79.3)	0.018*	0.043*
STR	6 (20.7)		

KPS Karnofsky performance status score, *GTR* gross total excision, *STR* subtotal excision, *PFS* progression-free survival, *OS* overall survival

* Statistical significant

Using Pearson's correlation coefficients, a significant correlation was found between MRP5 expression and Ki-67 index ($p = 0.002$). ROC curve analysis gave a cutoff MRP5 value of 11 and a Ki-67 index of 30 as best predicting survival. Patients with MRP5 index exceeding 11% (7 cases) differed significantly from those with MRP5 index ≤11% (22 cases) and were associated with worse survival (10.5 vs. 18 months, respectively; $p = 0.0002$) (Fig. 1). There was a significant increase in PFS for patients with a MRP5 index lower than 11% ($p = 0.0085$). Patients with Ki-67 index lower than 30% had increased PFS ($p = 0.047$) and longer survival (15 vs. 11 months, $p = 0.0084$). Patients with gross total tumor excision had a median survival of 17 months, whereas in patients with subtotally excised tumors, the median survival time was 10.5 months (Fig. 2). The difference was statistically significant ($p = 0.043$). There was a significant increase in PFS for patients with gross total tumor excision ($p = 0.018$). The median survival for patients with KPS over 80 was 15 months, whereas for patients with KPS under or equal to 80, it was 12 months; however, the difference was not statistically significant. Similarly, no significant difference was observed between patient's age, gender and survival. In multivariate analysis, MRP5 index and extent of tumor resection were identified as factors with independent prognostic power ($p = 0.02$, 95% CI 1.38–52.35 and $p = 0.048$, 95% CI 1.01–10.28, respectively).

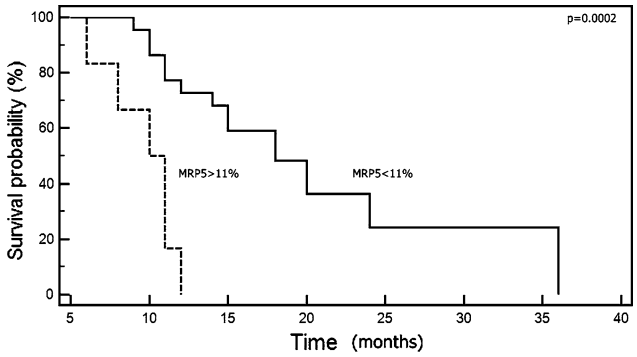

Fig. 1 *Graphs* showing the relationship between MRP5 index (cutoff = 11%) and survival in glioblastoma patients

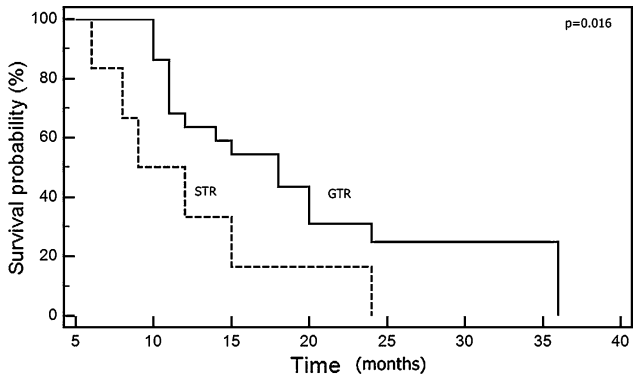

Fig. 2 *Graph* showing representative Kaplan–Meier survival curve of the patients grouped according to the extent of resection

Discussion

The present study demonstrated that the immunohistochemical expression of MRP5 may have a prognostic implication in patients with GBM. To the best of our knowledge, no other study has reported a correlation between MRP5 index and survival in patients with GBM. Furthermore, Ki-67 index lower than 30% and tumor's gross total excision were also associated with a better prognosis. Patients with preoperative KPS over 80 lived longer; however, this difference was not statistically significant, probably due to the limited number of patients.

Chemoresistance is a major obstacle for effective cancer treatment and can be present in a tumor at the time of initial diagnosis or can develop following treatment with chemotherapeutic agents [2]. Various genes have been implicated such as *MDR1*, *MRPs*, major vault protein (*MVP*) gene, the *MGMT* gene and the *Survivin* gene. These genes have been implicated toward conferring resistance to a wide range of chemotherapeutic drugs such as vincristine, temozolomide, etoposide and cisplatin [5, 6]. The *MDR1* gene encodes a

transmembrane Pgp that produces a broad pattern of resistance to several structurally and functionally unrelated drugs. Consequently, it reduces the intracellular drug concentration. P-gp efflux pump has been previously showed that is involved in resistance to temozolomide-mediated cytotoxicity. Schaich et al. [11] reported that the exon12 C1236T polymorphism of the *MDR1* gene turned out to be an independent prognostic factor in glioblastoma patients treated with temozolomide.

MRPs are members of the ABC superfamily of transmembrane proteins that act as ATP-dependent drug efflux pumps. Several chemotherapeutic agents are substrates; therefore, their accumulation is prevented [12, 18–20]. Nine MRP members have been identified so far. In gliomas, MRP1, 3, 4 and 5 have been reported to be expressed more than the other members [9, 10]. Benyahia et al. studied the MRP1 expression in surgical tumor samples from 17 patients with gliomas. MRP1 expression was observed in all tumor with more than 90% of stained tumor cells in 14/15 high-grade gliomas. MRP1 expression was also found at the membrane of the vascular endothelial cells. Furthermore, these authors reported that indomethacin, a MRP's inhibitor in addition to its other anti-glioma mechanisms [21], increased the cytotoxic effect of vincristine and etoposide in glioma cell lines [22]. Jin et al. [23] detected increased MRP1 and reduced MRP3 gene expression in glioma stem-like cells, but upon differentiation of cells, they noticed the opposite phenomenon (reduction of MRP1 and increased MRP3 expression), suggesting that multidrug resistance may be regulated predominantly by MRP1 in stem cells but by MRP3 in more differentiated glioma cells. Calatozzolo et al. [13] found immunohistochemical expression of glutathione-S-transferase pi, MRP1, MRP5, Pgp and MRP3 in glioma cells. Recently, Kuan et al. [10] using real-time PCR and immunohistochemistry demonstrated that that the MRP3 gene is expressed both at the mRNA level and at the protein level in high-grade gliomas, but minimally in normal brain tissue. Patients with GBMs that expressed elevated MRP3 mRNA levels in tumor biopsy samples had a higher risk of death. Inhibition of multidrug resistance may result in an increased accumulation of an anticancer drug inside cancer cells, so that lower doses may be required to produce an equivalent toxicity. Several compounds have been reported to reverse the MDR status. Thus, elacridar and tariquidar, the latest Pgp inhibitors, are currently being evaluated in clinical trials since the earlier generation inhibitors were ineffective [7, 8]. Regarding MRPs, MRP3-specific antibodies are being explored as vehicles for radioisotopes or cytotoxic drugs to target MRP3-expressing cancer cells [24]. Similarly, according to our findings, MRP5 may be an additional useful tumor-associated antigen for future targeted therapies.

In summary, our results suggest that determination of MRP5 in surgical tumor specimens of GBM patients may hold prognostic significance. Although the present study has certain limitations due to small number of patients and use of only one method of protein expression, it warrants further investigation in a larger number of patients to explore the role of MRP5 as well as MRP1 and MRP3 in prognosis and in targeted therapies for patients with GBM.

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