

Prognostic genetic markers in malignant gliomas

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Abstract

Glioblastomas are the most frequent and malignant brain tumors in adults. Surgical cure is virtually impossible and despite of radiation and chemotherapy the clinical course is very poor. Epigenetic silencing of MGMT has been associated with a better response to temozolomide-chemotherapy. We previously showed that temozolomide increases the median survival time of patients with tumors harbouring deletions on 9p within the region for p15(INK4b), p16(INK4a), and 10q (MGMT).

The aim of this study was to investigate the methylation status of p15, p16, 14^{ARF} and MGMT in glioblastomas and to correlate the results with the clinical data.

Only patients with KPS > 70, radical tumor resection, radiation and temozolomide-chemotherapy after recurrence were included.

We observed promoter methylation of MGMT in 56% (15/27) and of p15 in 37% (10/27) of the tumors, whereas methylation of p16 and p14ARF were rare.

Interestingly, methylation of p15 emerged as a significant predictor of shorter overall survival (16.9 vs. 23.8 months, $p=0.025$), whereas MGMT promoter methylation had no significant effect on median overall survival under this treatment regimen (22.5 vs. 22.1 months,

$p=0.49$). In the presence of other clinically relevant factors, p15 methylation remains the only significant predictor ($p=0.021$; Cox regression).

Although these results need to be confirmed in larger series and under different treatment conditions, our retrospective study shows clear evidence that p15 methylation can act as an additional prognostic factor for survival and underlines that this tumor suppressor, involved in cell cycle control, can act as an attractive candidate for therapeutic approaches in glioblastomas.

Keywords: glioblastoma, O(6)-methylguanine-DNA methyltransferase (MGMT), methylated MGMT, temozolomide

Introduction

Glioblastomas [World Health Organization (WHO) grade IV] are the most frequent and the most malignant brain tumors in adults. They arise either de novo without recognizable precursor lesions or develop from lower grade astrocytomas (secondary GBM). Despite of multimodal therapy approaches, the prognosis is generally poor but varies markedly between the malignancy grades and even between individuals with the same malignancy grade. Less than half of the

patients survive more than a year. (1) Besides radical surgery, a higher preoperative Karnofsky Performance Score (KPS) and younger age are predictors of a more favourable clinical course. (2-5)

Over the past decades, genetic abnormalities involved in pathogenesis and progression of these tumors were identified. Several alterations were also shown to be correlated with prognosis.

In novel studies progression-associated genetic markers in differing tumor entities as well as in gliomas are increasingly observed. Because of the mostly focal appearance of the changes to start with, the molecular-cytogenetic methods with their high dissolving ability are on this occasion essential. In a study which has just been finished, we compared the genetic aberration profile of the diffuse gliomas with the response rate of temozolomide chemotherapy. Because of the observation, we assume that there is minority of patients who survive for longer than 5 years after the recognition of a glioblastoma, surgery and a combined radio- and chemotherapy. We could show an until now unknown correlation of the patterns of genetic changes with the varying response rate of patients on this chemotherapy. Using CGH and LOH we could prove that gliomas patients with a tumor which shows a loss of 9p or 10q significantly benefit from temozolomide treatment. The gene for the O(6) - methylguanine-methyltransferase (MGMT) is localised on chromosome 10 (10q26) and encoded as the DNA repair-enzyme O(6) -alkylguanine-DNA-alkyltransferase (OGAT). The function of OGAT is the protection of the cells from alkylating substances by the removal of damaging DNA-methylisations i.e. for the demethylation of methylguanine. The

effect of the alkylating cytostaticum temozolomide is based exactly on these damaging DNA methylisations. The effect of the chemotherapeuticum is therefore impeded by MGMT and a loss of this would because of this be of advantage for the treated patients. Interesting for secondary studies is the question if the appropriate chromosomal regions on the apparently intact second chromosome 10 are mutated, methylated or still active. Therefore the expression status and the methylation pattern of MGMT in the post-characterised gliomas be analysed on a DNA-level. For that reason it should be clarified if the MGMT gene on the apparently intact chromosome 10 is still active or if it has been made inactive by the promotor-hypermethylation and if a reduced dose of the gene will already be sufficient for therapeutic success. Current studies on anaplastic astrocytomas and glioblastomas have shown that patients with a promotor hypermethylation of the MGMT gene benefit significantly from a nitrosourea substance and temozolomide chemotherapy in comparison to patients whose tumors do not show any methylation in the MGMT gene (27, 28).

Deletion or mutation of the p16(INK4a)/ARF/p15(INK4b) locus on chromosome 9p21 is among the most common alterations seen in human cancer and in human gliomas. (6-9) The INK4a locus encodes two gene products that are involved in cell cycle regulation through inhibition of CDK4-mediated RB phosphorylation (p16) and binding to MDM2 leading to p53 stabilization (p14^{ARF}). The tumor suppressor gene products p16 and p15 are both capable of binding to CDK4 and CDK6, these kinases associate with D-type cyclins and these

binary complexes are responsible for phosphorylation of RB-protein at mid G1 of the cell cycle. The phosphorylation of RB is assumed to be critical for progression through G1 and entry into S-phase of the cell cycle. Binding of INK4 to CDK4/6 inhibits its kinase activity and thereby arrests progression through the cell cycle in mid-late G1. (10)

Over half of the high grade gliomas lack a functional INK4a/ARF locus. Gliomas with intact INK4a/ARF carry mutations in other components of the RB and p53 pathways implicating these two pathways as being absolutely critical in cell growth and death control. (11-14) Previous studies showed that deletion of 9p including the INK4a/b locus is a significant unfavorable prognostic factor for survival of glioblastoma patients. (15, 16) Further on, this alteration has been reported to be inversely correlated with the chemosensitivity of malignant gliomas. (17)

Over the past years aberrant DNA methylation was shown to be a common molecular lesion in human tumors as well, which had also impact on patient prognosis and treatment response. Epigenetic silencing of p16 and p15 was shown in a variety of human neoplasms, in glioma patients hypermethylation is reported in about 30% of the cases. (18-21) Whereas in other tumors inactivation of both tumor suppressor genes was associated with prognosis and response to chemotherapy, a prognostic and predictive role in gliomas is not shown. (22-25)

On the other hand, epigenetic silencing of the DNA repair gene O6-methylguanine-DNA methyltransferase (MGMT) on chromosome 10q26 by hypermethylation has been linked to a better prognosis for glioblastoma patients

treated with alkylating agents like temozolomide. (26-29) A benefit was not observed for patients with MGMT promoter methylation and BCNU-chemotherapy. (30) Another study comparing different treatment regimens showed that the prognostic effect was only significant when patients were treated simultaneously with radio - and chemotherapy. (31) These results suggest that the reported impact of MGMT methylation is strongly dependent on therapeutic modalities and schedules.

In our previous study we identified the negative prognostic impact for deletions on 9p and 10q, which can be compensated by temozolomide treatment. (16) In the current setting, our aim was to investigate the methylation status of p15, p16, p14^{ARF} and MGMT, and correlate the results with the clinical data of the glioblastoma patients.

Materials and Methods

Patients and tumor samples

The retrospective study included 27 glioblastoma patients (23 primary and 4 secondary GBM) who underwent surgery at the Department of Neurosurgery of the Saarland University, Homburg, Germany. After radical tumor surgery, all patients received standard radiation therapy (RT) (1.8-2 Gy, total dose of 60 Gy) and adjuvant temozolomide chemotherapy in case of recurrence. The doses were 150 mg/m² for 5 days in 4 week cycles. Specimens of resected tumor were immediately shock frozen in liquid nitrogen and stored at -80°C or fixed in formalin and embedded in paraffin. All patients gave written informed consent for the use of the tumor samples for genetic analysis.

Methylation-specific polymerase chain reaction (MS-PCR)

DNA of the tumor samples was isolated following standard protocols with chloroforme followed by sodium bisulfite modification. Promoter hypermethylation of the MGMT, p15, p16 and p14^{ARF} genes were determined by MS-PCR as described previously. (18, 26, 32) The amplified products were electrophoresed on 3.5% agarose gels and visualized with ethidium bromide. Methylated blood DNA was included in each PCR set as methylated and unmethylated controls, respectively.

Statistical analyses

Comparison of survival times between

groups defined by methylation status was performed by Kaplan-Meier curves and with two-sided log rank tests. Multivariate Cox regression analysis was performed to identify significant predictors for survival. Effects in these models were quantified by hazard ratio estimates with 95% confidence intervals. Median survival rates were calculated using the Kaplan-Meier method.

Results

Clinical Data

Median age at surgery was 49 years (range 26-70), the sex ratio was 2.375 (19 men / 8 women) (Table 1) and median post operative KPS was 100 (range 80-100, 18 patients with KPS 100).

Case	Histologic type	age/sex	ST [months]	MGMT-status	p15-status	p16-status	p14 ^{ARF} -status
T4789	GBM	38/m	55,3	M	U	U	U
596/98	GBM	32/m	21,1	U	U	U	U
1099/98	GBM	49/m	23,8	U	U	U	U
1782/98	sGBM	26/m	24,2	M	U	U	U
896/99	GBM	51/m	13	U	M	U	U
1326/99	GBM	56/m	19,2	U	U	U	U
1349/99	GBM	54/m	72,6	M	U	U	U
1460/99	GBM	31/f	11,9	M	M	U	U
1795/99	sGBM	31/m	6,9	M	U	U	U
265/00	GBM	46/f	23,1	U	M	n.a.	U
497/00	GBM	58/f	16,9	M	M	U	U
1106/00	GBM	70/m	19,8	M	M	U	U
1691/00	sGBM	39/f	80,2	M	U	U	U
1707/00	GBM	47/m	29,4	U	U	U	U
643/01	GBM	54/f	26,6	U	U	U	U
662/01	GBM	70/m	13,4	U	M	U	U
1405/01	GBM	53/m	7,4	U	M	M	U
6/02	GBM	41/m	26,2	M	U	U	U
369/02	sGBM	37/m	20,2	M	U	U	U
947/02	GBM	46/m	17,7	M	M	U	U
1536/02	GBM	53/m	15,1	U	U	U	U
1940/02	GBM	54/f	14,3	M	M	U	U
XXL/02	GBM	45/m	8,6	M	U	U	U

316/03	GBM	63/f	23,7	U	U	n.a.	U
784/03	GBM	52/m	22,5	M	U	U	U
831/03	GBM	58/m	53	U	U	n.a.	U
1457/03	GBM	37/m	49,1	M	M	n.a.	U

Methylation analyses

The MGMT promoter was methylated in 15/27 cases (55.6%), the p15 promoter was methylated in 10/27 (37%) glioblastomas. All secondary GBM (4/4) showed a methylated MGMT promoter and an unmethylated p15 promoter. Methylation of p14^{ARF} was absent in all (27/27) investigated GBM. Methylation status of p16 was available for 23/27 glioblastomas. Hypermethylation of p16 was detected in only 1/23 cases (4.3%) (Table 1).

Clinical outcome

Overall median survival was 22.5 months with a two-year survival rate of 35.0%.

In univariate analyses, MGMT methylation had no impact on overall survival (22.5 vs. 22.1 months, $p=0.49$, log-rank test, Fig. 1A), whereas p15 methylation was associated significantly with a shorter overall survival (16.9 vs. 23.8 months; $p=0.0252$, log-rank test, Figure 1B). Table 2 contains estimated hazard ratios and p -values for univariate analyses for all examined variables.

We also performed a multivariate analysis including parameters previously identified as significant. As shown in Table 3, only p15 methylation emerged as a significant prognostic factor after adjusting for KPS, sex, age and MGMT. In the first analysis, the predictors KPS and age enter as numerical variables in the model, in the second analysis KPS and age are dichotomized with cutoffs 90 and 50 in order to reduce model complexity. The

gender variable sex is set to 1 for females and 0 for males. Both analyses yield very similar results, identifying p15 methylation as only significant predictor.

Discussion

A better understanding of the genetic alterations predicting disease outcome and therapy response in patients with high grade gliomas will help to optimize both treatment and overall outcome. In our study setting, MGMT promoter methylation had no significant impact on survival, in contrast with other studies that observed a strong effect on patient response and on survival in larger cohorts treated with radio- and temozolomide-chemotherapy. (26, 28) This discrepancy might result, besides our smaller patient cohort, from the different treatment schedules. Actually, comparing various treatment modalities in glioblastomas, the prognostic effect of MGMT methylation was observed only when simultaneous chemoirradiation was administered. (26, 31)

Further on, our PCR-results showed besides the methylated band also unmethylated bands in a large number of tumors. Taking into account this heterogeneity and that we are dealing with diffusely growing gliomas, (33, 34) this observation arises most likely from a different tumor cell population and from normal cells contaminating the tumor sample. Nevertheless, this heterogeneous methylation pattern may also result in different amounts of MGMT and therefore affect chemotherapy response.

All evidence collected to date implicates that the INK4a/ARF gene products are critically important in control of growth arrest and senescence. Loss of p16 and ARF expression is associated with many human cancers, particularly gliomas. (20) A number of studies have shown that reconstitution of INK4a/ARF expression in glioma cells altered growth characteristics, reduced tumorigenicity and decreased invasive potential. These studies demonstrate the importance of the INK4a/ARF pathway in suppression of the neoplastic phenotype and suggest that restoration of a functional INK4a/ARF locus will be an important means of controlling the growth of gliomas. (36, 37)

A striking observation of our study is the significant correlation of p15 methylation with a poorer clinical course. Loss of p15 had not been widely investigated previously as a potential determinant of chemo- and radiosensitivity or as a prognostic factor. To our knowledge, this is the first study showing inactivation of p15 by promoter hypermethylation to be a predictor for an unfavorable clinical course. Cyclin dependent kinase inhibitors (p16, p21, p27) were shown to exhibit an antitumor effect in malignant gliomas inducing growth arrest and apoptosis in cell culture. (36, 37) Further on, retrovirus mediated transfer of INK4a halts glioma formation in a rat model. (38) This corroborates the idea that retrovirus mediated gene transfer of INK4a/b may also be an effective means to arrest human gliomas. Therefore, restoring the normal function of p15 by gene therapy is an attractive goal in the treatment of human gliomas.

Furthermore, our finding that glioblastomas have not simultaneously hypermethylated the investigated tumor

suppressor genes on 9p implicates that these tumors carry no general defect in their pattern of CpG island methylation. Interestingly, all secondary GBM (4/4) showed a methylated MGMT promoter and an unmethylated p15 promoter which indicates that distinct molecular pathways constitute for primary and for secondary glioblastomas and let them differ both in biological behavior and in clinical outcome. This corresponds to the finding that methylation of p14^{ARF} is associated with a shorter patient survival and is mutually exclusive of MGMT promoter methylation except of one case in low grade astrocytoma who underwent progression or recurrence. (35) In our patient cohort that mainly consists of primary glioblastoma, p14^{ARF} was not observed, supporting that this alteration is mainly restricted to secondary glioblastoma and therefore in the pathway of astrocytoma progression.

Although these results need to be confirmed in larger series, our retrospective study suggests that p15 hypermethylation can act as an additional important prognostic factor for survival in glioblastomas. Further investigations have to clarify if p15 methylation is a predictive factor for temozolomide treatment response and can act as a prognostic parameter for survival, independent of therapy.

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