p15 promoter methylation - A novel prognostic marker in glioblastoma patients

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Abstract. Glioblastomas are the most frequent and malignant brain tumors in adults. Surgical cure is virtually impossible and despite 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, p14^{ARF}* and *MGMT* in glioblastomas (n=27) 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% and of *p15* in 37% of the tumors, whereas methylation of *p16* and *p14^{ARF}* 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). Although these results need to be confirmed in larger series as well as under different treatment conditions, our retrospective study shows clear evidence that *p15* methylation is an important prognostic factor for survival and underlines that this tumor suppressor, involved in cell cycle control, is an attractive candidate for therapeutic approaches in glioblastomas.

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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 favorable 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.

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 cyclines 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

Table I. Patient characteristics and methylation status of MGMT, p15, p16 and $p14^{ARF}$.

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

GBM, glioblastoma multiforme; sGBM, secondary glioblastoma multiforme; m, male; f, female; ST, survival time; M, methylated; U, unmethylated; n.a., not available.

been reported to be inversely correlated with the chemosensitivity of malignant gliomas (17).

Over recent 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 (9,22-24).

On the other hand, epigenetic silencing of the DNA repair gene *O^6-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 (25-28), whereas no benefit was observed for patients with MGMT promoter methylation and BCNU-chemotherapy (29). Another study comparing different treatment regimens showed that the prognostic effect was only significant when patients were treated simultaneously with radio- and temozolomide-chemotherapy (30). These results suggest that the reported impact of *MGMT* methylation is strongly dependent on therapeutic modalities and schedules.

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

Patients and methods

Patients and tumor samples. The retrospective study included 27 glioblastoma patients (23 primary and 4 secondary GBM)

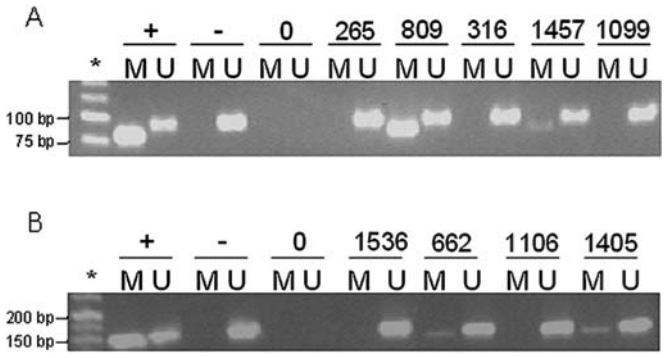

Figure 1. Methylation specific PCR of: (A) MGMT; and (B) p15 promotor in GBMs. *, Molecular size marker; U, unmethylated DNA; M, methylated DNA; +, positive control; -, negative control; 0, blank value.

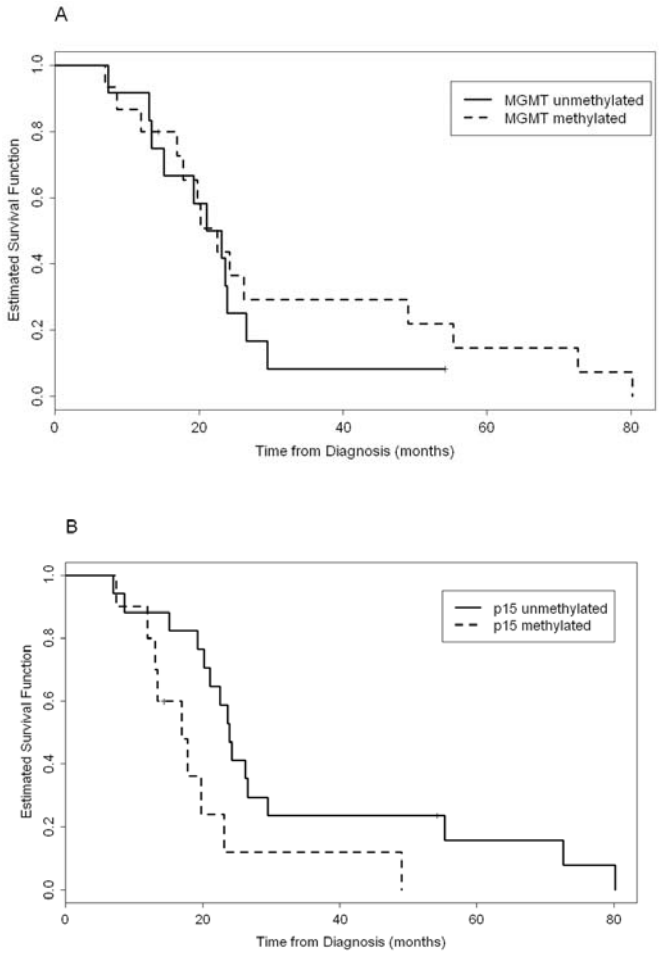

Figure 2. Kaplan-Meier survival curves. Overall survival of: (A) *MGMT* methylation; and (B) *p15* methylation. Censored data (patients still alive) are plotted as hash marks.

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/m^2 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 for histopathological diagnosis. The patients gave written informed consent for the use of the tumor samples for genetic analysis.

Methylation-specific polymerase chain reaction (MS-PCR). DNA from fresh frozen tumor samples was isolated following standard protocols with chloroform followed by sodium bisulfite modification. Promoter hypermethylation of the *MGMT, p15, p16* and *p14^{ARF}* genes were determined by MS-PCR as described previously (18,25,31). 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 I) and median post-operative KPS was 100 (range 80-100, 18 patients with KPS 100).

Methylation analysis. The *MGMT* promoter was methylated in 15/27 cases (55.6%), the *p15* promoter was methylated in 10/27 (37%) glioblastomas (Fig. 1). 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 I).

Clinical outcome. Overall median survival was 22.5 months with a 2-year survival rate of 35.0%. One patient (831/03) was alive at end of follow-up with a survival time of 53 months and one patient (case 1940/02) was lost of follow-up.

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

We also performed a multivariate analysis including all parameters used in the univariate models. As shown in Table IIB, 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 cut-offs 90 and 50 in order to reduce model complexity. The gender variable sex is set

Table II. Overall survival in glioblastoma patients.

Variable	Hazard ratio	95% CI	SD	p-value
A, Univariate Cox-regression analysis				
KPS	1.089	0.983-1.21	0.052	0.095
KPS ≥90	0.214	0.025-1.83	1.100	0.121
Sex	0.826	0.325-2.10	0.476	0.687
Age	1.014	0.973-1.06	0.021	0.508
Age ≤50	0.813	0.362-1.83	0.414	0.617
MGMT methylation	0.744	0.320-1.73	0.431	0.490
p15 methylation	2.655	1.100-6.42	0.451	**0.025**
B, Multivariate Cox-regression analysis				
KPS	1.067	0.963-1.18	0.0521	0.210
Sex	0.636	0.238-1.70	0.5018	0.370
Age	1.015	0.972-1.06	0.0222	0.500
MGMT methylation	0.638	0.240-1.70	0.4982	0.370
p15 methylation	2.705	1.010-7.25	0.5027	**0.048**
Multivariate Cox-regression analysis with dichotomous variables				
KPS ≥90	0.668	0.0635-7.02	1.201	0.740
Sex	0.682	0.2544-1.83	0.503	0.450
Age ≤50	0.810	0.3371-1.95	0.447	0.640
MGMT methylation	0.615	0.2388-1.59	0.483	0.310
p15 methylation	3.198	1.1871-8.62	0.506	**0.021**

KPS, Karnofsky Performance Score; CI, confidence interval; SD, standard deviation.

to 1 for females and 0 for males. Both analyses yield very similar results, identifying p15 methylation as the 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. Our study confirms the fact that the prognostic significance of *MGMT* promoter methylation depends strongly on the treatment regimen. Actually, comparing various treatment modalities in glioblastomas, the prognostic effect of *MGMT* methylation was observed only when simultaneous chemoirradiation was administered and not when temozolomide was administered adjuvant after tumor recurrence as in our study setting (25,27,30,32).

Further on, our PCR-results showed besides the methylated band also unmethylated bands in a large number of tumors (Fig. 1). Taking into account the heterogeneity of these diffusely and infiltrating 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 which would be interesting to investigate in a larger patient cohort.

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 (35,36).

The striking observation of our study is the significant correlation of *p15* methylation with a poorer clinical course. Loss of *p15* has 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 (35,36). Moreover, retrovirus-mediated transfer of *INK4a* halts glioma formation in a rat model (37). 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 might be an attractive goal in the treatment of human gliomas.

Furthermore, a general defect in their pattern of CpG island methylation can be excluded because the investigated glioblastomas have not simultaneously hypermethylated the investigated tumor suppressor genes on 9p. Interestingly, all secondary GBM (4/4) showed a methylated *MGMT* promoter and an unmethylated *p15* promoter which underlines that distinct molecular pathways constitute the primary and the secondary glioblastomas and are responsible for their different biological behavior and in clinical outcome. This corresponds to the finding that methylation of *MGMT* promoter is mutually exclusive of $p14^{ARF}$ methylation and the latter is associated with a shorter patient survival, except in one case of low grade astrocytoma who underwent progression or recurrence (38).

In our patient cohort, $p14^{ARF}$ methylation was not observed. Hence our cohort mainly consists of primary glioblastomas, a larger sample size of secondary glioblastomas has to be analyzed to clarify if this alteration is mainly restricted to secondary glioblastoma and therefore is an important step 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 reliable prognostic parameter for survival, independent of therapy.

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