Promoter methylation of *RB1*, *P15*, *P16*, and *MGMT* and their impact on the clinical course of pilocytic astrocytomas

CHRISTOPH SIPPL[1], STEFFI URBSCHAT[1], YOO JIN KIM[2], SEBASTIAN SENGER[1], JOACHIM OERTEL[1] and RALF KETTER[1]

[1]Department of Neurosurgery; [2]Institute of Neuropathology, University of Saarland, Faculty of Medicine, Homburg/Saar, D-66424 Homburg, Germany

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Abstract. Promoter methylation of *P15*, *P16*, RB transcriptional corepressor 1 (*RB1)* and O-6-methylguanine-DNA methyltransferase (*MGMT)* impacts the prognosis of numerous glioma subtypes. However, whether promoter methylation of these genes also has an impact on the clinical course of pilocytic astrocytoma remains unclear. Using methylation-specific polymerase chain reaction, the methylation status of the tumor suppressor genes *P15*, *P16*, *RB1*, and *MGMT* in pilocytic astrocytomas (n=18) was analyzed. Immunohistochemical staining for the R132H mutation of the isocitrate dehydrogenase (NADP(+)) 1, cytosolic (*IDH1)* gene was performed. Clinical data including age, gender, localization of tumor, extent of resection, treatment modality, progression-free survival and overall survival were collected. The methylation index for *P15*, *P16*, *RB1* and *MGMT* was 0.0, 0.0, 5.6% (1/18) and 44.5% (8/18), respectively. If the *MGMT* promoter was methylated, the probability of relapse and second subsequent therapy was significantly increased (P=0.019). The one patient with methylation of *P15* demonstrated a poor clinical course. The pilocytic astrocytomas of all 18 patients revealed wild-type *IDH1*. Clinically, there was a significant correlation of subtotal resection with the occurrence of relapse (P=0.005) and of the localization of the tumor with the extent of resection (P=0.031). Gross total resection was achieved significantly more often in pediatric patients than in adult patients (P=0.003). Adult patients demonstrated more relapses following the first tumor resection (P=0.001). The present study indicates that methylation of *MGMT* is associated with a poor clinical course and represents an age-independent risk factor for an unfavorable outcome. Other influential factors of outcome were the age of the patient and extent of resection.

Correspondence to: Dr Steffi Urbschat, Department of Neurosurgery, University of Saarland, Faculty of Medicine, Geb. 90 Kirrbergerstr, Homburg/Saar, D-66424 Homburg, Germany
E-mail: steffi.urbschat@uks.eu

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Introduction

Brain tumors are the most common solid neoplasms in children (1). Pilocytic astrocytomas (PAs), as WHO grade I neoplasias, represent up to 20% of brain tumors in children and adolescents (2). They are found in the hypothalamus, periventricular region of the third ventricle, and cerebellum (3). They generally have a relatively benign clinical course, with a 10-year survival rate of 95% (4). This good prognosis is primarily because PAs are usually sharply circumscribed, and thus, they can often be completely resected. Hence, surgery is the gold standard and represents the preferred therapy (5,6). In addition, these tumors show only a slight tendency to infiltrate healthy tissues (7). Nonetheless, some PAs show a more malignant course, particularly in adult patients (8,9).

The pathognomonic molecular characteristic of PAs in pediatric patients is a KIAA1549-BRAF fusion transcript, resulting from a somatic duplication of 7q34. Mutations of the proto-oncogene B-Raf (BRAF V600E mutation) are found in less than 10% of tumors (10,11). However, additional genetic alterations can be present in the relatively uncommon case of PAs in adult patients. The main genetic alterations in PAs in adult patients is a KIAA1549-BRAF fusion transcript, found in 20-32% of cases; *FGFR1* mutation; and the absence of BRAF V600E mutation (12-17). Moreover, *IDH1* R132H mutation might play a more important role in adult PAs (18,19). In case of *NF1* mutation, PAs may involve the optic pathways, optic nerve, and chiasm (12,14). A review of the literature on adult PAs has shown that most cases remain genetically uncharacterized. Therefore, the question remains whether additional molecular markers can be found at an epigenetic level to help predict the clinical course of the disease. The best studied epigenetic modification is DNA methylation. In this process, methyl groups are covalently attached to CpG islands in the promoter regions of genes by DNA methyltransferase, resulting in the suppression of transcription. These CpG islands exist in approximately 40% of the promoter regions found in humans. However, not all CP dinucleotides are CpG islands that can be methylated. The methylation status of *P15*, *P16*, *RB1*, and *MGMT* has been shown to be important in the oncogenesis of WHO grade II-IV gliomas. *P15*, *P16*, and *RB1* play a crucial role in the cell cycle as tumor suppressors and influence progression and prognosis in glial tumors (20). P15 and

p16 can bind and therefore inhibit CDK4 and CDK6. Inactive CDK4 and CDK6 are responsible for the hypophosphorylated status of RB1, resulting in cell arrest (21). Therefore, p15 and p16 act as tumor suppressors in the late G1 phase (22). Mutations of and deletions in *RB1*, *P15*, and *P16* are among the most frequently observed genetic alterations in glial tumors and can result in a more aggressive biological behavior of the tumor (23-26).

MGMT is a DNA repair protein that removes alkyl groups and adducts at the O6 position of guanine. It protects healthy cells against mutagenic effects, and loss of expression due to *MGMT* promoter hypermethylation has been proposed as a predisposing factor for the acquisition of TP53 transition mutations in oncogenesis (27). *MGMT* hypermethylation is associated with a significantly shorter progression-free survival (PFS) in patients with breast cancer and low-grade astrocytomas (28-31). MGMT can also protect cells with high-grade astrocytomas against the cytotoxic effects of alkylating chemotherapeutic agents (32). The question arises whether specific methylation patterns of these genes also correlate with the clinical course of PAs as WHO grade I neoplasias. We hypothesize that in PAs, promoter methylation of *P15*, *P16*, *RB1*, and *MGMT* results in a higher frequency of relapses with a reduced PFS and overall survival (OS). Furthermore, we expect to find different specific methylation patterns in adult and pediatric PAs.

Materials and methods

Patients. In this retrospective study, tumor tissues from patients who underwent surgery at the Saarland University Medical Center in Homburg between 1999 and 2014 and who had clinical data available from January 1999 to December 2016 were used. Individual follow-up periods ranged from 4 months to 14.7 years. Inclusion criteria were a neuropathological diagnosis of PAs (WHO grade I) and a sufficient amount of tumor tissue for DNA isolation. NF1 mutation was not detected in any tumor specimen. No included patient had a tumor at the optic nerve. This study was approved by the local Ethical Review Board, and written informed consent was obtained from all patients or their representatives (Ärztekammer des Saarlandes, Ethikkommission, No. 93/16). All procedures performed in this study were in accordance with the ethical standards of the 1964 Helsinki declaration. Genomic DNA was extracted from the resected tumor tissue. All tissue samples were stored at -80˚C.

Methylation analysis. DNA isolation was performed using a DNA isolation kit (Qiagen, QIAamp DNA Mini kit 50). The methylation status of promoter regions of *P15*, *P16*, *RB1*, and *MGMT* was determined by methylation-specific polymerase chain reaction. Therefore, 500 ng DNA of each tumor specimen as well as appropriate control samples were treated with bisulfite (Zymo Research, EZ DNA Methylation-Gold kit 200) (33). In summary, unmethylated cytosine was converted to uracil, whereas methylated cytosine remained unchanged. The modified DNA was recovered by ethanol precipitation and suspended in polymerase chain reaction (PCR) grade water. For analyzing the methylation status, the primer sequences listed in Table I were used (34-36). PCR was performed using a 25-μl reaction volume and 38 PCR cycles. All PCR products were electrophoretically separated on a 2% agarose gel. As a positive control, a chemically globally methylated DNA was used (Zymo Research, bisulfite-converted Human DNA). Genomic DNA isolated from a non-neoplastic dura mater tissue served as a negative control. In addition, each PCR included a control without any DNA template. An example of PCR results is presented in Fig. 1.

IDH1-R123H staining. Immunohistochemistry was conducted on 4-μm-thick formalin-fixed, paraffin-embedded tissue sections mounted on StarFrost Advanced Adhesive slides (Engelbrecht, Kassel, Germany). This was followed by drying at 80˚C for 15 min. Immunohistochemistry was performed on a BenchMark Ultra immunostainer (Ventana Medical Systems, Tucson, AZ, USA). Sections were stained with anti-IDH1-R132H antibody H09 (Dianova, Hamburg, Germany) as previously described (37).

Statistical analysis. All samples were scrutinized comparing the methylation status of *P15*, *P16*, *RB1*, and *MGMT* for the determining the PFS, OS, and occurrence of relapse. In addition, other clinical data such as age at onset, gender, tumor location, and treatment modality were collected. The Kaplan-Meier and log-rank test were used to calculate the PFS and OS in relation to promoter methylation. For statistical evaluation of the age at onset t-test for independent samples was applied. For the analysis of gender, tumor location, and treatment modality, a chi-square test was used. The significance level used in all tests was $P<0.05$. SPSS v. 21 was used as the statistical program.

Results

A total of 18 patients (12 males and 6 females) met the inclusion criteria. The most frequent localizations were the cerebellum (12 patients), medulla oblongata and cervical spine (3 patients), and cerebrum (2 patient). In one patient, the tumor was localized in the brainstem. The mean age at diagnosis was 17.9±15.8 years, ranging from 3.1 to 61.1 years. The mean follow-up duration was 4.9±4.2 years, with a range from 4 months to 14.7 years. There were six patients with an age at onset between 25.2 and 61.1 years; there were categorized as adult patients. The other 12 patients had disease onset between 3.1 and 18.4 years; they were categorized as pediatric patients (38,39). Table II shows an overview of collected data. Primary therapy after diagnosis was tumor resection in all patients. Gross total resection (GTR) was possible in nine patients. In the other nine patients, only subtotal resection (STR) was possible because of localization or infiltration of the tumor in eloquent areas of the brain. The extent of resection was determined by magnetic resonance imaging within 48 h postoperatively. Disease relapse occurred in six patients. These patients underwent a second surgery, with additional radiotherapy in two patients.

The PAs of all 18 patients were analyzed for promoter methylation of *P15*, *P16*, *RB1*, and *MGMT*. The methylation index (MI) of *P15*, *P16*, *RB1*, and *MGMT* was 0.0, 0.0, 5.6% (one patient, case 56/04), and 44.5% (8/18) (Fig. 2). Because no methylated promoter of *P16* and *RB1* was found, no further statistical analysis regarding these two genes was conducted. Promotor methylation of *P15* was found in one patient; however,

Table I. Primer sequences for methylation specific-polymerase chain reaction.

Author, year	Gene name	Forward (5'-3')	Reverse (5'-3')	Methylation	Length, (bp)	Temperature (˚C)	(Refs.)
Felsberg, 2009	MGMT	GTTTTTAGAACGTTTTGCGTTTCGAC	CACCGTCCCGAAAAAAAACTCCG	Methylated	122	54	(34)
		TGTGTTTTTTAGAATGTTTTGTGTTTTTGAT	CTACCACCATCCCAAAAAAAAACTCCA	Unmethylated	129	56	
Wong, 2000	p15	GCGTTCGTATTTTGCGGTT	CGTACAATAACCGAACGACCGA	Methylated	148	60	(35)
		TGTGATGTGTTTGTATTTTGTGGTT	CCATACAATAACCAAACAACCAA	Unmethylated	154	60	
Herman, 1996	p16	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	Methylated	150	65	(36)
		TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	Unmethylated	151	65	
Simpson, 2000	RB1	GGGAGTTTCGCGGACGTGAC	ACGTCGAAACACGCCCCG	Methylated	172	55	(37)
		GGGAGTTTTGTGGATGTGAT	ACATCAAAACACACCCCA	Unmethylated	172	55	

Figure 1. An example of methylation-specific polymerase chain reaction products for methylation of *MGMT* after electrophoretic separation. bp: base pairs, L: ladder, M: aberrant promoter methylation, and U: no aberrant promoter methylation. From left to right: No template control (water control), positive control (global bisulfite-converted DNA), negative control (DNA isolated from a healthy dura mater tissue), and several tumor samples. Tumor samples 1405/05 and 584/13 showed promoter hypermethylation of *MGMT*.

statistical analysis did not seem useful as only one such patient was observed. However, this patient with methylation of *P15* had the only fatal clinical course in the present cohort. The patient (case 56/04) showed relapse with local metastasis 4 months after the first surgery. A second tumor resection with subsequent chemotherapy (carboplatin + VCR) was unsuccessful, and the patient died 6 months after the first diagnosis.

If the *MGMT* promoter was methylated, relapse and second subsequent therapy occurred significantly more often (P=0.019; Fig. 3). If the methylation status of *MGMT* was used as a predictor for second therapy due to relapse, 77.8% of all patients could be correctly classified (binary logistic regression, P=0.016). When more closely examining the six patients with relapse, a huge difference in PFS between the patients with and those without methylation was found. One patient with relapse (case 1333/99) showed an unmethylated *MGMT* promoter. The PFS of that patient was 85.2 months. The other five patients showing relapse with a methylated *MGMT* promoter had an average PFS of 11.5 months.

There was no significant association between the age of patients and a specific pattern of methylation. Adult patients displayed a significant correlation with the non-cerebellar location of PAs. Patients with a non-cerebellar tumor localization were significantly older (38.5±17.08 years) at disease onset than those with a cerebellar localization (11.41±7 years; P=0.01).

There was a significant correlation between the extent of resection and occurrence of relapse (chi-square test, P=0.005). If only STR was achieved, relapse was more likely. In adult patients, STR was significantly more common (P=0.003). Adult patients showed significantly more relapses after the first tumor resection than pediatric patients (P=0.001). There was also a trend that methylation status of *MGMT* correlated with the frequency of STR (P=0.058).

However, a direct relation between age at disease onset and methylation status of *MGMT* could not be found. Age, gender, and localization of the tumor were not associated with the methylation status of *MGMT*. The PAs of all 18 patients had wild-type *IDH1*. An *IDH1*-R123H mutant could not be demonstrated in any tumor.

Discussion

PAs represent up to 20% of brain tumors in children and adolescents and are usually not malignant (2). However, some

Table II. Clinical characteristics of the patients.

Case	Sex	Age (years)	PFS (years), then relapse	Therapy	Localisation	Methylation status of RB1	p15	p16	MGMT
1646/05	M	25.2	0.9	STR+STR	Cerebellum	0	0	0	1
357/01	M	10.7	No rel	GTR	Cerebellum	0	0	0	0
04/14	M	13.8	No rel	GTR	Cerebellum	0	0	0	0
236/06	M	3.3	No rel	GTR	Cerebellum	0	0	0	0
56/04	F	34.9	0.3	STR+(STR with C)	Cervical spine	0	1	0	1
1176/00	F	3.1	No rel	GTR	Cerebellum	0	0	0	1
1333/99	M	61.1	7.1	STR+(STR with RT)	Medulla oblongata	0	0	0	0
236/07	F	11.5	No rel	GTR	Cerebellum	0	0	0	0
2184/13	F	49	2.0	STR+(STR with RT)	Brainstem	0	0	0	1
1940/00	M	4.1	No rel	GTR	Cerebellum	0	0	0	0
740/02	M	11.1	No rel	GTR	Cerebellum	0	0	0	1
1917/05	M	7.5	No rel	GTR	Cerebellum	0	0	0	0
1594/99	F	26.8	No rel	STR	Right lat. ventricle	0	0	0	0
119/04	M	4.6	No rel	GTR	Cerebellum	0	0	0	0
203/09	F	7.4	No rel	STR	Cerebellum	0	0	0	0
1850/05	M	13.3	1.3	STR+STR	Cervical spine	0	0	0	1
585/13	M	46.1	0.3	STR+STR+STR	Right lat. ventricle	0	0	0	1
1405/05	M	18.4	No rel	STR	Cerebellum	0	0	0	1

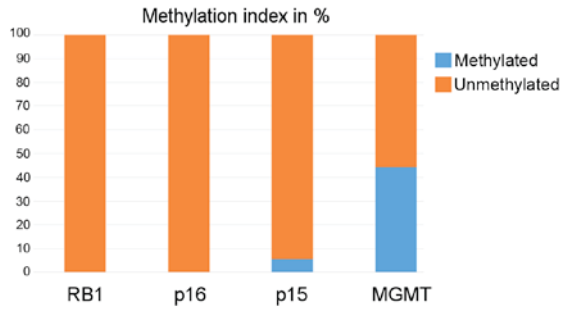

Figure 2. Presentation of the methylation index (in %) of different genes. Blue represents the methylated cases and orange the unmethylated cases. *RB1*=0.0%; *P16*=0.0%; *P15*=5.6% (1 case, case 56/04); *MGMT*=44.5% (8/18).

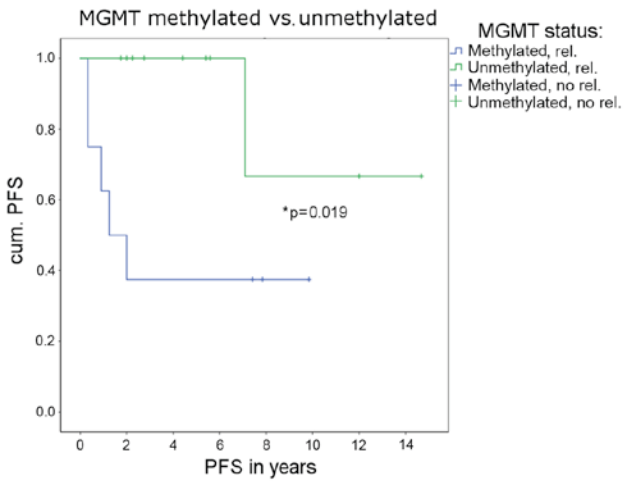

Figure 3. Kaplan-Meier curve of progression-free survival in relation to the methylation status of *MGMT* in years. (m=methylated, blue and u=unmethylated, green). The X-axis represents the PFS in years, and the y-axis represents the cumulative PFS. rel.=tumor relapse.

PAs show a more aggressive clinical behavior, particularly in adult patients (8,9). This trial aimed to identify new epigenetic markers to predict the course of PAs. If these predictors are available, patients could be stratified for an optimized follow-up. Because of their known impacts on glial tumors, the analysis focused on *P15*, *P16*, *RB1*, and *MGMT* in correlation with patients' clinical courses.

RB1 and *P16* showed no promoter methylation. The promoter of *P15* was methylated in one patient. This is consistent with the results of Uhlmann *et al* and Gonzales-Gomez *et al* who described PAs as not commonly methylated (40,41). However, their trials described only methylation profiles without the correlation of clinical parameters or further stratification of patients for age. A remarkable case in the present study was a patient with a PA at the cervical spine (case 56/04) with promoter methylation of *P15*. Despite GTR, local recurrence with meningeal metastases occurred. A second tumor resection with subsequent chemotherapy was unsuccessful, and the patient died 2 months later. Previous studies have shown that loss of expression, resulting from deletion or methylation of *P15*, is associated with a significantly worse prognosis for survival in glioblastomas (20,42). It is possible that promoter methylation of *P15* in this patient resulted in very aggressive tumor behavior and poor clinical course.

The presented results regarding promoter methylation of *MGMT* disproved the hypothesis that PAs are generally unmethylated. The PAs of all 18 patients had an *MGMT* MI of 44.5%. This remarkably high frequency of methylation of *MGMT* in PAs has not been reported in the literature thus far. Nevertheless, loss of MGMT expression because of promoter hypermethylation of the *MGMT* gene is a well-documented phenomenon in high-grade brain tumors (43,44). In the present study, patients showing tumors with promoter methylation of *MGMT* showed a significantly higher risk of relapse and necessity of secondary treatment. A closer look at the six patients with relapse revealed that when *MGMT* was methylated, the PFS was reduced. Studies on WHO grade II astrocytomas demonstrated that methylation of *MGMT* can be associated with a significantly shorter PFS (28). This supposes a higher malignancy in PAs if the *MGMT* promoter is methylated. A higher malignancy in patients having tumors with hypermethylation of *MGMT* vs. a lower malignancy in patients having tumors with unmethylated *MGMT* has also been demonstrated in breast cancer (28-31). In glioblastoma multiforme, the hypermethylation of *MGMT* is a well-known marker for better response characteristics than alkylating chemotherapy, resulting in a better prognosis (32). This does not contradict the findings in the present trial in PAs because none of the patients underwent alkylating chemotherapy.

In PAs, different genetic characteristics between adult and pediatric patients are known. Although a KIAA1549-BRAF fusion transcript is dominant in pediatric patients, in adults, *FGFR1* mutation and the absence of BRAF V600E mutation can also be found (12-17). In other recent investigations, an *IDH1* R132H mutation was described solely in adult patients (18,19). Therefore, the hypothesis was that methylation patterns are differently distributed between adult and pediatric patients. This was not the case in the present trial. *RB1* and *P16* were not methylated in adult or pediatric patients. Because of the low number of promoter methylations of *P15*, no reasonable conclusion can be drawn. The correlation between methylation of *MGMT* and occurrence of relapse was independent of age. However, adult patients displayed a significant correlation with the non-cerebellar localization of PAs. Tumor specimens of the included patients were scrutinized for analyzing *IDH1* R132H mutation. All patients showed wild-type *IDH1*, suggesting that *IDH1* R132H mutation in PAs is a rare event in adult patients.

In this trial, tumor recurrence was significantly more likely in cases of STR than in cases of GTR. This underlines the huge importance of radical surgery for PAs. Alford *et al* presented a similar correlation in a patient cohort with 51 PAs (38).

The main limitation of this trial is the low number of included patients. Hence, data in this trial should be critically scrutinized. With only 18 patients included, we acknowledge that the generalization of the results might be limited. Nevertheless, the results show that even in benign tumors, stratification based on molecular markers is becoming increasingly important. In the present trial, methylation of *MGMT* was a significant age-independent predictor of the necessity of a second therapy. Consequently, a further evaluation of epigenetic markers in larger cohorts of patients with PAs under the special aspect of *MGMT* is recommendable. Though speculative, a further idea is to assess methylation of *MGMT* in fluid probes obtained

by liquid biopsy (45). The proof of principle has already been furnished in colorectal cancer (46). In cases of tumors of the central nervous system, such as PAs, the cerebrospinal fluid next to blood samples could be used. This could enable a prognosis even before surgery.

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