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Methylation Profiling Identifies Epigenetic Markers for High-grade Gliomas

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Abstract. *Hypermethylation of CpG island is an epigenetic event prevalent in human gliomas. Here we employed a high-throughput microarray approach for a global search of DNA methylation to identify novel epigenetic loci in specific glioma subtypes. Hierarchical clustering analysis separated 20 glioma samples according to their WHO histopathological subtypes – pilocytic astrocytomas (PAs, grade I), oligoastrocytomas (OAs, grade II) and glioblastomas (GBMs, grade IV), based on their unique methylation patterns. The overall methylation frequency of the low-grade PAs was significantly less than that of the more aggressive OAs and GBMs (0.45% versus 2.0% and 1.4%; PAs versus OAs, $p<0.01$; PAs versus GBMs, $p<0.01$). The lower level of DNA methylation observed in PAs may be in part due to the increased methylation of multiple CpG islands which occur in more advanced tumors. However, the young age of onset of PAs may also contribute to this observed difference. Although there were many hypermethylated loci exclusive to the OA and GBM subtypes, the methylation frequencies between these groups were not significantly different. Analysis by methylation-specific PCR on an expanded set of samples and on more glioma subtypes further confirmed an epigenetic marker, SMARCA5, the hypermethylation of which was preferentially observed in grade IV, but not in grades I or II gliomas ($p<0.0001$). The intermediate grade III gliomas showed low levels of SMARCA5 hypermethylation. Epigenetic loci uncovered in the present study recapitulate the histopathological differences of these gliomas, indicating that these molecular changes may be responsible for the development of the different glioma subtypes. On-going work in our laboratory has shown that some of these loci are indeed hypermethylated in the early stages of astrocytic tumors.*

Hypermethylation of CpG island is an epigenetic phenomenon frequently observed in solid tumors (1). The epigenetic event usually occurs at 5'-ends of genes and is associated with inactive chromatin configuration that prevents the process of transcription initiation (2). Increasing evidence indicates that, in addition to genetic mutations or deletions being responsible for cancer development, this methylation-mediated gene silencing can play a causal role in tumorigenesis. In gliomas as well as other central nervous system tumors, several genes have been reported to be hypermethylated. These include *TP73* (3), *MGMT* (4), *CDKN2A* (5), *CDKN2B* (6), $p14^{ARF}$ (7), *Peg3* (8), *RB1* (9), *hMLH1* (10), *THBS1* (11), *N33* (12), *ERα* (12), *HIC1* (13), *c-abl* (12) and *c-fos* (14). Using restriction landmark genomic scanning, Costello *et al.* (15, 16) conducted gene-by-gene and global surveys, and their results suggest that hypermethylation of CpG islands is widespread in gliomas. One implication is therefore the potential use of this epigenetic information for diagnosis and prognosis of different glioma entities. Indeed, a further study by Esteller *et al.* (17) has suggested that hypermethylation of the *MGMT* CpG island is a useful predictor of responsiveness of gliomas to alkylating agents.

In the present study, we used differential methylation hybridization (DMH) (18) to conduct global analysis of DNA methylation and to identify novel epigenetic markers for specific glioma subtypes. DMH is a high-throughput microarray approach in which a total of 7,776 short CpG island tags, known as probes, were arrayed on microscopic

Abbreviations: DMH, differential methylation hybridization; PAs, pilocytic astrocytomas; OAs, oligoastrocytomas; GBMs, glioblastomas.

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Key Words: DNA methylation, differential methylation hybridization, gliomas, CpG island microarray, *SMARCA5*.

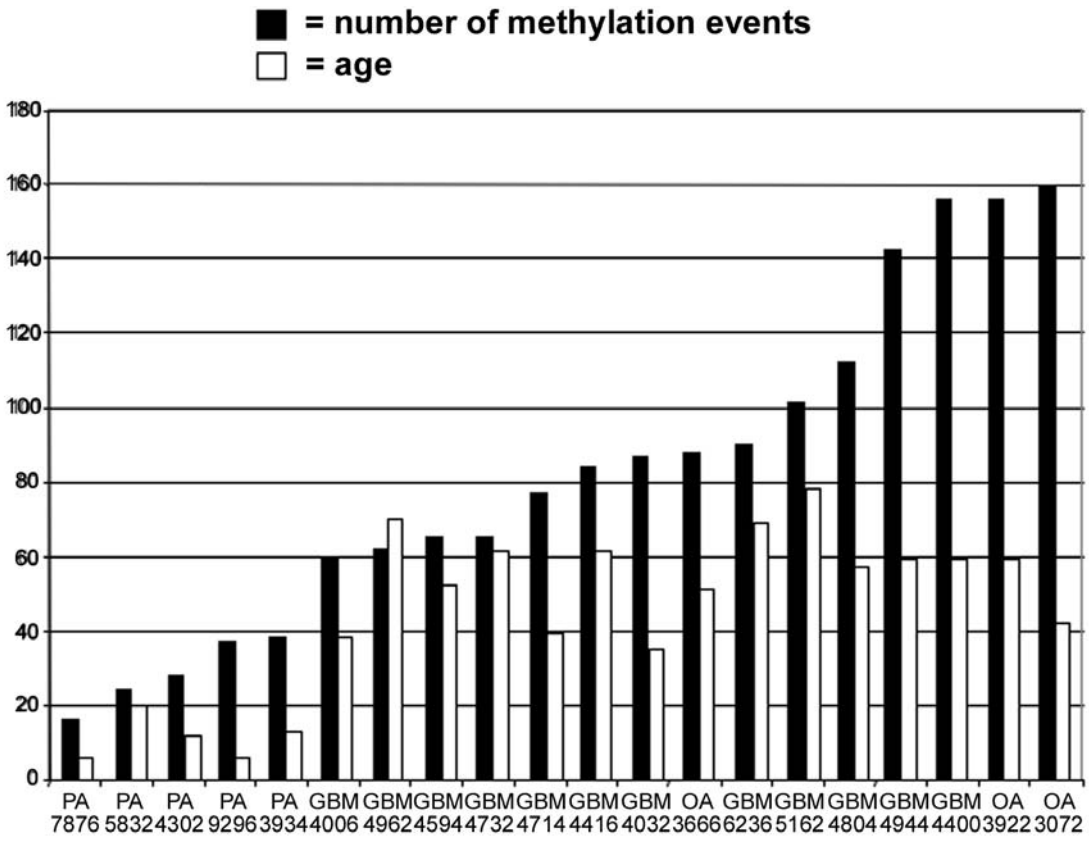


Figure 1. *Methylation scores in pilocytic astrocytoma (PA), oligoastrocytoma (OA), and glioblastoma (GBM). This diagram shows the overall methylation events detected by differential methylation hybridization (DMH) in 20 glioma samples (black columns). White columns are the age at diagnosis.*

slides. DNA targets representing pools of methylated CpG island fragments were prepared from glioma samples or normal controls and used in microarray hybridization. Differential hybridization intensities of CpG islands identified in gliomas relative to their controls reflected methylation differences in these loci. Cluster analysis further uncovered loci that were preferentially hypermethylated in the more advanced tumors and distinguished gliomas according to their histological classification. These loci may harbor potential epigenetic biomarkers for disease diagnosis and prognosis.

Materials and Methods

Tumor samples. Clinical specimens were obtained from glioma patients treated at the University Hospital of Bonn, Germany. The age of the patients ranged from 5 to 79 years. H & E stainings were performed on histological sections, and necrotic lesions and adjacent normal tissue were removed from the corresponding frozen sections to enrich the tumor component of glioma specimens. DNA was extracted using standard protocols with proteinase K digestion and phenol/chloroform extraction. White matter samples from 4 epilepsy patients (ages 35-48) who underwent temporal lobe resection served as controls.

Differential methylation hybridization (DMH). The DMH protocol was performed as described (18). To generate targets, 2 μg genomic DNA from tumor and control samples were digested with *Mse*I (New England Biolabs) and purified using PCR purification columns (Qiagen). The digests were ligated to 0.5 nmol of unphosphorylated linkers (H24: 5'-AGG CAA CTG TGC TAT CCG AGG GAT and H12: 5'-TAA TCC CTC GGA) using T4 DNA ligase (New England Biolabs) and were subsequently digested with two methylation-sensitive restriction enzymes (*Bst*UI and *Hpa*II). This was followed by 20 cycles of amplification with the H24 linker primer using DeepVent$^{(exo-)}$ polymerase (New England Biolabs). Amplified products of tumor and control samples were purified and labeled with fluorescent dyes Cy5 and Cy3, respectively. Labeled DNAs from tumor and control samples were co-hybridized to a microarray slide containing 7,776 CpG island genomic fragments (on average 500-bp) (18). Post-hybridization washing step was carried out at 50˚C with 1X SSC, 0.1% SDS followed by an additional wash in the same solution at room temperature and a last wash with 0.2X SSC at room temperature. Slides were dried by centrifugation and scanned with a GenePix 4000A scanner (Axon Instruments) for data acquisition. Images were analyzed with the GenePix Pro 3.0 software. Each spot was localized using a grid and the repeat-containing CpG loci were excluded from further analysis. Signal intensities for single-copy sequences were normalized and loci having Cy5 to Cy3 ratio

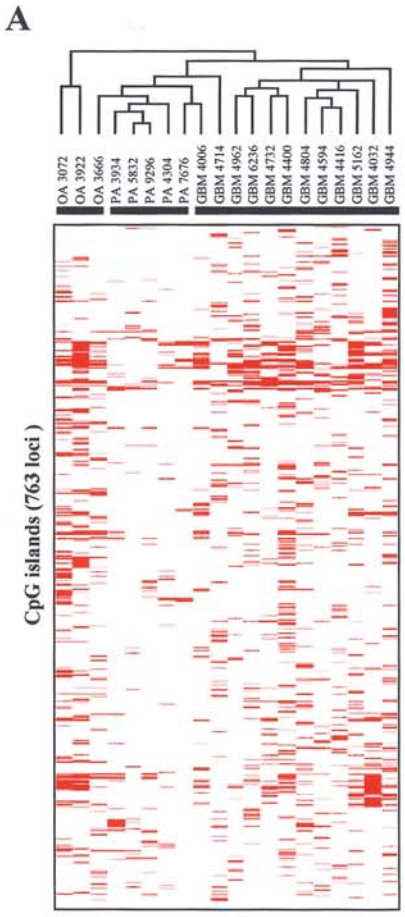
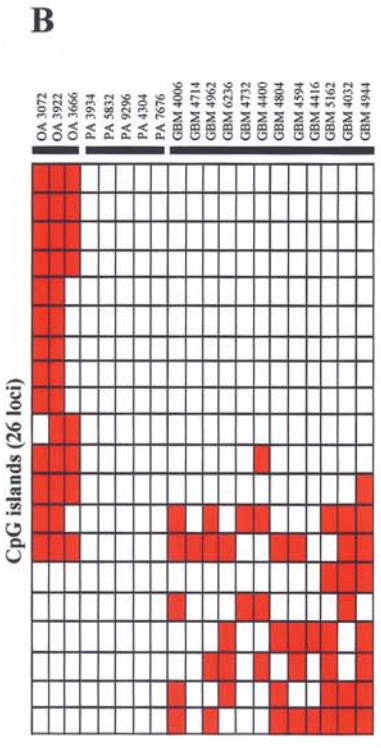


Figure 2. *A, Hierarchical clustering of hypermethylated CpG islands in 20 glioma subtypes. The dendrogram (at the top) lists the degree of relatedness among tumors. The row corresponds to each of the 763 hypermethylated loci. B, Subpanel of CpG island loci that preferentially hypermethylated in oligoastrocytomas (OAs) or glioblastomas (GBMs), but not in pilocytic astrocytomas (PAs).*



Clone ID	Chromosome	Gene Bank	Gene Name	Description
787	15q22.31	XM_372576		Similar to RIKEN cDNA C230094B15
396	14q23.1			No match
519	14q32.12	AF070587		CpG island, 5' region of AF070587
721	19q13.2	NM_016457	*PRKD2*	protein kinase D2
798	16p13.3	NM_178844	*NOD3*	NOD3 protein (NOD3), mRNA.
470	17q25.3	AJ312320	*Ov/Br septin*	CpG island 5' of *MSF* ovarian/breast septin epsilon
641	15q23			No match
804	8p23.1	BC043573		clone IMAGE: 5222470, mRNA
785	16q12.1	NM_002968	*SALL1*	CpG island, 3' of homo sapiens sal-like 1
790	16q11.2	BC001344		CpG island 5' of XTP3-transactivated protein A
788	1q32.1	AK027656		Kelch-like protein C3IP1
789	17p13.1	BC011371	*CHRNB1*	Cholinergic receptor, nicotinic, beta polypeptide 1, mRNA
233	9p11.2	AB095935	*AGP2*	CpG island 5' of ankyrin repeat domain 18A
159	6q25.3	AF318616	*SYNJ2*	CpG island 5' of synaptojanin 2 promoter
783	18q12.1	U46745	*DTNA*	Human dystrobrevin-beta mRNA
203	11q13.3	AF264779	*C11orf23*	Chromosome 11 open reading frame 23
489	4q31.21	BC023144	*SMARCA5*	SWI/SNF related, regulator of chromatin
792	7p15.2	BC013971	*HOXA10*	Homeo box A10
808	19q13.32	NM_012155	*EML2*	Echinoderm microtubule associated protein like 2
325	1p31.3	NM_012183	*FoxD3*	CpG island 5' of forkhead box D3

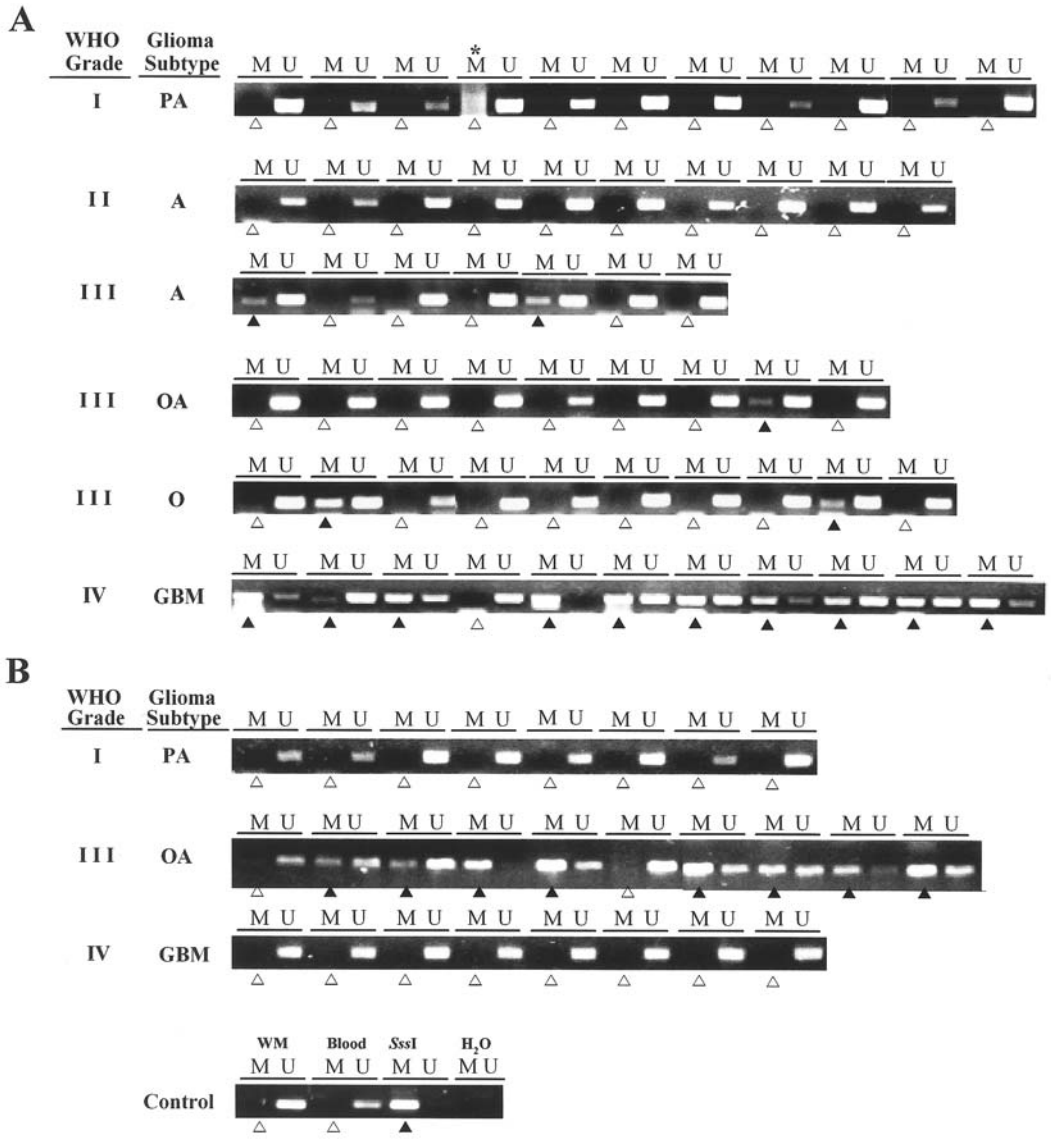


Figure 3. *Methylation-specific PCR. Bisulfite-treated DNA was amplified with specific methylated (M) or unmethylated (U) primers for CpG #489 (Panel A) and for CpG #519 (panel B), respectively. PCR products were separated on 2% agarose gels. PA: pilocytic astrocytomas; A: anaplastic astrocytomas; OA: oligoastrocytoma; O: oligodendroglioma; GBM: Glioblastomas; WM: white matter as a negative control; and SssI: M. SssI methylase-treated control. △: methylation-negative and p: methylation-positive. ▲: This lane shows an overloaded sample, smears present the trailing of primer-dimer.*

cut-off ratios ≥1.5 were scored as hypermethylated. This method was previously used to identify hypermethylated loci in several tumor types (19). Data were then exported and subjected to unsupervised cluster analysis using Cluster 3.0, and the results of the hierarchical clustering were visualized using Treeview.

Methylation-specific PCR. Genomic DNA was bisulfite-modified as described (20), which leaves methylated cytosines intact but converts all unmethylated cytosines to uracil and subsequently to thymine residue upon PCR amplification. Briefly, 1μg DNA was denatured by 2M NaOH and then deaminated in the presence of hydroquinone and sodium bisulfite. Bisulfite-treated DNA was then desalted using the Wizard Plus DNA Purification system (Promega) and purified for methylation-specific PCR (21). PCR primers unique to bisulfite-converted DNA were designed to discriminate between the methylated and unmethylated sequences of a CpG island locus and PCR products were amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems). Primers for the methylated allele of CpG #519 were: 5'-ttt tag ttt aac ggg gtt cgt tag c (sense strand) and: 5'-tta acc cga aaa acc cta cgt aac g (antisense strand); for the unmethylated allele were: 5'-ttt agt tta atg ggg ttt gtt agt (sense strand) and: 5'-taa ccc aaa aaa ccc tac ata aca (antisense strand). Methylated primers for CpG #489 were: 5'-gcg gga tta gcg ttg gtc gta gac g (sense strand) and: 5'-cgc aaa cga

aac atc ata tcg tcc g (antisense strand); and unmethylated primers were: 5'-tgt ggg att agt gtt ggt tgt aga t (sense strand) and 5'-cac aaa caa aac atc ata tca tcc (antisense strand). Peripheral blood lymphocytes and white matter were used as negative controls, and universal methylated DNA (Intergen) was used as positive control. PCR products were separated on a 2% agarose gel and visualized with the Gel-Doc 100 Imaging System (BioRad).

Statistical analysis. Statistical analysis was conducted using the SigmaStat 2.0 program.

Results and Discussion

DMH was used to globally profile CpG island hypermethylation in 20 gliomas and 4 normal (white matter) controls. These gliomas were categorized into three subtypes defined by the World Health Organization (WHO) according to tumor behavior and histology: pilocytic astrocytoma (PA)-grade I, oligoastrocytoma (OA)-grade II, and glioblastoma (GBM)-grade IV (22). Of the 7,776 CpG islands screened by DMH, 763 loci were identified to be hypermethylated (*i.e.*, normalized Cy5/Cy3 $\geq$1.5) in at least one glioma sample relative to the control. Excluding $\sim$15% of repeat sequences present in the microarray panel, the overall methylation frequency of the individual tumors ranged from 0.3 to 2.4% (18 to 160 hypermethylated loci). As shown in Figure 1, the overall methylation event is significantly less in the low-grade PAs as compared to the more aggressive OAs and GBMs (0.45% *versus* 2.0% and 1.4%; PAs *versus* OAs, $p<0.01$; PAs *versus* GBMs, $p<0.01$). Since PA is usually seen in younger patients (ages 5 to 20) and OA or GBM in older patients (ages 38-78), the present observation is consistent with the notion that there can be an age-related factor to CpG island hypermethylation (12).

Hierarchical clustering of the 763 methylated loci clearly separated the 20 gliomas based on their clinicopathological parameters and WHO grading (Figure 2A). A subpanel of 20 loci hypermethylated in advanced gliomas (OAs and GBMs) was selected for sequence analysis (Figure 2B). Within the subpanel, 8 loci were preferentially methylated in GBMs, six of which contained sequences identical to known genes (*AGP2, Cllorf23, SMARCA5, HoxA10, EML-2*, and *FoxD3*.) To substantiate this microarray finding, methylation-specific PCR was conducted in an expanded set of gliomas (PAs, analplastic astrocytomas, oligodendroglioma, grade III OAs, and GBMs). We focused the analysis on a CpG island fragment, CpG#489, the sequence of which matched to the promoter and the first exon of *SMARCA5*, a known chromatin modulator (23). As shown in Figure 3A, hypermethylation of *SMARCA5* was observed in 91% (10/11) of grade IV and 20% (5/26) of grade III, respectively. No hypermethylation of this locus was seen in low-grade (grade I or II) gliomas (0/21). Taken together, these results indicate that hypermethylation of *SMARCA5* is closely associated with high-grade GBMs. Since hypermethylation of multiple CpG island loci is known to occur in more advanced cancers (15), future study will be conducted to identify additional epigenetic markers for high-grade gliomas.

The subpanel shown in Figure 2B also reveals at least 10 CpG island loci uniquely hypermethylated in the OA (grade II) group. Among these, the sequences of 4 loci matched to known genes or cDNAs, including *PRKD2, MSF, MGC5627*, and *C31PI.* Since only 3 OAs were examined in the DMH analysis, we verified the cluster analysis result on a locus, CpG #519, by methylation-specific PCR using an expanded panel of all 3 types of gliomas. In line with the microarray data, hypermethylation of this locus was detected in 80% (8/10) of OAs, but in none of the PAs (0/8) or GBMs (0/8) samples analyzed (Figure 3B). Although CpG #519 was mapped to a CpG island fragment of no known function, this fragment is located on chromosome14q32, a genomic region reported to be lost in 21% of the OAs examined (24). It is therefore conceivable that this chromosomal region may contain a critical tumor suppressor, the expression of which can be inactivated *via* genetic and/or epigenetic alteration in OAs. Future studies will be performed to identify the gene associated with this CpG island locus and to determine the functional consequence of its methylation-mediated gene silencing in the development of this glioma subtype.

The present analysis provides proof of principle that CpG island hypermethylation can be a potential source of biomarkers for different gliomas. Current classification of central nervous system tumors is based on the origin of putative tumor cells and histopathological features according to the WHO grading system. These classification and grading systems, however, can be subjective and there are considerable variations in treatment outcome in patients with similar histopathological features. Data from this and other studies point to the need for a comprehensive understanding of genetic and epigenetic alterations underlying all forms of central nervous system tumors to facilitate tumor diagnosis. The DMH approach we describe here allows for global screening of epigenetic alterations that may be uniquely present in different forms of gliomas. As such, the epigenetic markers uncovered by this approach may complement current histopathological classification for disease diagnosis and may lead to future disease treatment as DNA methylation is a potentially reversible process.

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