RESEARCH ARTICLE DOI 10.1111/j.1750-3639.2007.00083.x

DNA hypermethylation and Aberrant Expression of the *EMP3* Gene at 19q13.3 in Human Gliomas

Annegret Kunitz[1]*; Marietta Wolter[2]*; Jörg van den Boom[2]; Jörg Felsberg[2]; Björn Tews[3]; Meinhard Hahn[3]; Axel Benner[4]; Michael Sabel[5]; Peter Lichter[3]; Guido Reifenberger[2]; Andreas von Deimling[6]; Christian Hartmann[7]

[1] Department of Neuropathology, Charité, Universitätsmedizin Berlin, D-13353 Berlin, Germany.
Departments of [2] Neuropathology, [5] Neurosurgery, Heinrich Heine University, D-40225 Düsseldorf, Germany.
[3] Division of Molecular Genetics (B060), [4] Central Unit Biostatistics (C060), [6] Department of Neuropathology, Ruprecht-Karls Universität, D-69120 Heidelberg, Germany. [7] Clinical Cooperation Unit Neuropathology (G380), Deutsches Krebsforschungszentrum.
*These authors contributed equally to this work.

Corresponding author:
Christian Hartmann, MD, Clinical Cooperation Unit Neuropathology, Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Germany
(E-mail: *christian.hartmann@dkfz.de*)

Allelic losses on 19q are found in the majority of oligodendroglial tumors and approximately one-third of diffuse astrocytomas. However, the tumor suppressor genes (TSG) on 19q are still elusive. Using cDNA microarray expression profiling, *EMP3* at 19q13.3 was among those genes showing the most pronounced expression differences. In line with this, other authors reported *EMP3* as being epigenetically silenced in neuroblastomas and astrocytomas. To further investigate *EMP3* as a TSG candidate on 19q13.3, we performed molecular analysis of this gene in 162 human gliomas. Mutation analysis did not reveal *EMP3* alteration in 132 gliomas. In oligodendroglial tumors, we found that aberrant methylation in the 5′-region of *EMP3* was significantly associated with reduced mRNA expression and LOH 19q. In astrocytomas, *EMP3* hypermethylation was also paralleled by reduced expression but was independent of the 19q status. *EMP3* hypermethylation was detected in more than 80% of diffuse, anaplastic astrocytomas and secondary glioblastomas. Primary glioblastomas, however, mostly lacked *EMP3* hypermethylation and frequently overexpressed *EMP3*. Our data corroborate that oligodendroglial and astrocytic gliomas often show *EMP3* hypermethylation and aberrant expression. Furthermore, our findings suggest that primary and secondary glioblastomas are not only characterized by distinct genetic profiles but also differ in their epigenetic aberrations.

Brain Pathol 2007;17:363–370.

INTRODUCTION

Allelic losses affecting the long arm of chromosome 19 (19q) are frequently found in oligodendroglial and astrocytic tumors (29, 30, 40). In oligodendroglial tumors, including oligodendrogliomas World Health Organization (WHO) grade II (OII), anaplastic oligodendrogliomas WHO grade III (OIII), oligoastrocytomas WHO grade II (OAII) and anaplastic oligoastrocytomas WHO grade III (OAIII), 19q losses are already frequent in the low-grade (WHO grade II) tumors indicating a role early in tumor development (9, 30). In contrast, deletions of 19q are more commonly detected in anaplastic astrocytomas WHO grade III (AIII) as compared with diffuse astrocytomas WHO grade II (AII), suggesting a progression-associated role in these tumors (38–40). Another difference between astrocytic and oligodendroglial tumors consists in the extent of 19q losses and their association with losses on 1p. The vast majority of oligodendroglial tumors demonstrate deletions of the entire 19q arm while interstitial and smaller telomeric deletions are more frequent in astrocytomas (9, 30). Furthermore, 19q deletions in oligodendroglial tumors usually occur in combination with deletions of the short arm of chromosome 1 (1p), whereas such co-deletions are rare in astrocytomas (10, 30, 40). The different deletion patterns may be explained by the frequent presence of unbalanced t(1;19)(q10;p10) translocations in oligodendroglial but not astrocytic tumors (7, 15).

Approximately 50% of the mixed oligoastrocytomas carry the oligodendroglioma-associated 1p and 19q deletions while approximately 30% contain *TP53* mutations, which is a hallmark alteration in diffuse astrocytic gliomas (23, 30). The most malignant astrocytic tumor, glioblastoma multiforme (GBM), also exhibits 19q deletions at more than random frequency. However, 19q deletions are less frequent in primary glioblastomas (pGBM), which develop *de novo* with a short clinical history, than in secondary glioblastomas (sGBM), which develop by progression from a pre-existing lower grade glioma (24). Combined 1p and 19q deletions are rare in both types of glioblastoma (24, 40).

Patients with oligodendroglial tumors exhibiting combined 1p and 19q losses have a more favorable clinical outcome when compared with those patients whose tumors have retained both chromosomal arms (3, 36). The reasons for the better prognosis of patients with combined 1p and 19q losses are still unknown. Furthermore, the relevant tumor suppressor genes (TSG) on both chromosome arms have not been identified yet. Allelic deletion studies hinted towards a smallest region of overlapping deletions at 19q13.3; however, mutation analyses of TSG candidates from this region failed to detect any mutations (9). Therefore, epigenetic alterations, in particular aberrant methylation of CpG sites in the 5′-CpG-rich region of putative TSG, have become a major focus of interest. A few genes on 19q have been reported as being aberrantly methylated and transcriptionally silenced in 19q-deleted glio-

mas. These include the *ZNF342* zinc finger transcription factor gene at 19q13 (13) and the maternally imprinted, paternally expressed gene *PEG3* at 19q13.4 (34). In glioma cell lines, expression of both genes could be restored by the treatment with 5-aza-2′-deoxycytidine (13, 20). However, 19q losses in oligodendroglial tumors affect maternal and paternal alleles in a random fashion (8), which argues against a major role of imprinted genes like *PEG3* in these neoplasms.

Recently, the epithelial membrane protein 3 gene (*EMP3*) at 19q13.3 has been reported to show frequent promoter methylation in high-grade astrocytomas and in neuroblastomas (1), which both are tumor entities showing frequent allelic deletions at 19q13.3 (22, 29, 40). *EMP3* expression could be restored by 5-aza-2′-deoxycytidine treatment of neuroblastoma cell lines and reintroduction of *EMP3* into these cell lines resulted in lower colony formation *in vitro* as well as in reduced tumor growth in nude mice, thus indicating a tumor-suppressive function of EMP3 protein in neuroblastomas (1). Furthermore, *EMP3* promoter methylation was associated with a less favorable clinical outcome in neuroblastoma patients (1). Independent of these data, we identified *EMP3* as an interesting candidate gene in a microarray-based expression profiling of gliomas with and without losses on 1p and 19q, which revealed *EMP3* as the gene with the highest linear expression ratio differences between 1p/19q-deleted vs. non-deleted gliomas (32). The precise physiological function of the EMP3 protein has not been resolved yet. Its homology to PMP22 suggests a role in cell proliferation and apoptosis (31). To further investigate the significance of *EMP3* aberrations in human gliomas, we performed a systematic molecular analysis of this gene in a large series of oligodendroglial and astrocytic gliomas.

MATERIAL AND METHODS

Tissue samples and DNA/RNA extraction. Human glioma tissue samples were collected at the Department of Neuropathology, Charité, Berlin, and at the Department of Neuropathology, Heinrich Heine University, Düsseldorf. All samples were analyzed in an anonymized manner as approved by the local institutional review boards. Histological classification was performed according to the WHO classification of tumors of the nervous system (16). From each case, a tissue sample was snap-frozen immediately after operation and stored at −80°C. A tumor cell content of at least 80% was histologically determined for each specimen used for nucleic acid extraction. DNA and RNA extraction was performed as reported elsewhere (37).

Loss of heterozygosity analysis of chromosome 19q. Five microsatellite loci located on chromosome arm 19q (either *D19S433*, *D19S431*, *D19S718*, *D19S559* and *D19S601* or *D19S396*, *D19S219*, *D19S1182*, *D19S572* and *D19S210*) were analyzed for loss of heterozygosity (LOH) in each tumor using non-denaturing polyacrylamide gel electrophoresis and silver staining, as reported elsewhere (6, 11).

Real-time reverse transcription-PCR. RNA for *EMP3* expression analysis was available from 10 OII, 21 OIII, 2 OAII, 8 OAIII, 9 AII, 10 AIII, 9 pGBM and 9 sGBM. The mRNA expression level of *EMP3* was determined by quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) using the GeneAmp 5700 sequence detection system (Applied Biosystems, Darmstadt, Germany). Continuous quantitative measurement of the PCR product was enabled by intercalation of SYBR Green fluorescent dye to the double-stranded DNA. The transcript level of *EMP3* was normalized to the transcript level of *ARF1* (*ADP-ribosylation factor 1*, NCBI GenBank Accession-No. M36340). The primer sequences and PCR conditions for *EMP3* and *ARF1* are published elsewhere (1, 5). As reference tissue, we used commercially available adult human brain RNA (BD Biosciences, St. Jose, CA, USA) as well as RNA extracted from cerebral tissue samples of two different adult patients who were operated on for non-neoplastic lesions. One of the samples was obtained by surgery for brain trauma and the second one was obtained at autopsy from a patient who died from liver failure.

Single-strand conformation polymorphism (SSCP) analysis and direct sequencing. A total of 132 gliomas, including 28 OII, 33 OIII, 5 OAII, 31 OAIII, 7 AII, 15 AIII and 13 pGBM, were screened for *EMP3* mutations. Mutation analysis was carried out by SSCP analysis followed by direct DNA sequencing as described (10). In brief, all four coding exons of *EMP3* were amplified by PCR from genomic DNA. The PCR primer sequences were as follows: exon 2, *sense* 5′-tgccaacctct tgagactcc-3′ and *antisense* 5′-caagtatc caaaggggaaca-3′ (184 bp product); exon 3, *sense* 5′-tgaccctatcccctctcctt-3′ and *antisense* 5′-gggtttaccttccccctttga (212 bp); exon 4, *sense* 5′-gtgatgtccccctctgtgtc-3′ and *antisense* 5′-caattcttgggggattaggg-3′ (215 bp); exon 5, *sense* 5′-atgacgtgtggttttcatctt-3′ and *antisense* 5′-aaggaagatcaaggcagagc-3′ (300 bp). PCR products were screened for mutations by electrophoresis on 8% and 14% nondenaturing polyacrylamide gels. The SSCP band patterns were visualized by silver staining of the gels. PCR products with aberrant SSCP patterns were sequenced in both directions on an ABI Prism 377 DNA Sequencer (Applied Biosystems).

Sodium bisulfite sequencing of the EMP3 5′-CpG-rich region. A total of 162 gliomas, including 22 OII, 36 OIII, five OAII, 11 OAIII, 24 AII, 25 AIII, 30 pGBM and nine sGBM, as well as three non-neoplastic brain tissue samples were investigated for *EMP3* hypermethylation by sequencing of sodium bisulfite-modified DNA using the primers described by Alaminos et al (1). Sixteen CpG-sites were evaluated for methylation within a 341-basepair amplicon encompassing the *EMP3* transcription start site. Two different methods were applied. For cross-validation, six tumors were analyzed by both methods. In total, 89 tumors and three non-neoplastic control tissues were investigated by direct sequencing of PCR products obtained from sodium bisulfite-modified DNA. Two of the control tissues were derived from the temporal lobe (cortex and white matter) of two patients operated on for chronic epilepsy. The third control tissue was obtained by autopsy and corresponded to macroscopically and microscopically tumor-free occipital lobe tissue (cortex and white matter) from a patient who died of a frontal glioblastoma. Sodium bisulfite treatment of the DNA was carried out as described elsewhere (12). PCR was performed for 40 cycles using

HotStarTaq DNA polymerase (Qiagen, Hilden, Germany). PCR products were purified and directly sequenced by using the BigDye Cycle Sequencing kit v1.1 (Applied Biosystems). The second method, which was applied to 73 tumors, involved modification of the tumor DNA using the EZ DNA Methylation kit (Zymo Research, USA) according to the manufacturer's instructions and as described before (27). Following PCR amplification with the same set of primers, PCR products were either directly sequenced or cloned using the TOPO TA Cloning kit (Invitrogen, Karlsruhe, Germany). In the case of cloning, 10 white colonies were picked for each tumor and sequenced using M13 reverse primers and the BigDye Cycle Sequencing kit.

Scoring of the methylation data. The directly sequenced samples were scored according to the ratio of the cytosine to thymidine peak at each CpG site as follows: 0—no methylation; 1—weak methylation, ie, intensity of the methylated signal lower than 1/3 relative to the unmethylated signal; 2—moderate methylation, ie, intensity of the methylated signal between 1/3 and 2/3 relative to the unmethylated signal; 3—strong methylation, ie, intensity of the methylated signal higher than 2/3 of the unmethylated signal. Those cases that were analyzed by sequencing of cloned PCR products were scored as follows: 0—no methylation in any of 10 clones; 1—weak methylation, ie, methylation detected in 1–3 of 10 clones; 2—moderate methylation; ie, methylation detected in 4–6 of 10 clones; 3—strong methylation, ie, methylation deteted in 7–10 of 10 clones. Based on these semiquantitative scores, the tumors were subdivided into two groups: (1) no *EMP3* hypermethylation (methylation score 1, 2, or 3 in less than 50% of the 16 investigated CpG sites) vs. (2) *EMP3* hypermethylation (methylation score 1, 2 or 3 in ≥50% of the 16 investigated CpG sites). In addition, a numerical methylation score was calculated for each tumor by summing up the methylation levels (1 to 3) at each of the 16 investigated CpG sites.

Statistical analyses. Pairwise comparisons of *EMP3* methylation scores and *EMP3* expression levels between two independent groups were performed by using the *t*-test. Two-dimensional contingency tables were analyzed using Fisher's exact test. The log rank test was used for pairwise comparisons of survival time distributions. Survival curve estimation using the method proposed by Kaplan and Meier was performed with GraphPad Prism 4. For multivariable analysis of survival time data Cox's proportional hazards regression model was used including *EMP3* hypermethylation, 1p/19q loss, WHO grade and the patient age at operation. The multivariable analysis was done using R, version 2.4.1 (28). All reported *P*-values are two-sided. A result was judged as statistically significant at a *P*-value smaller or equal to 0.05.

RESULTS

Allelic losses on 19q. LOH at one or more microsatellite markers on 19q was detected in 16 of 22 OII (73%), 24 of 35 OIII (69%), three of five OAII (60%), five of 11 OAIII (45%), six of 20 AII (30%), eight of 23 AIII (35%), 15 of 30 pGBM (50%) and three of eight sGBM (37.5%) investigated by microsatellite analysis.

Mutation analysis of EMP3. SSCP analysis of the entire *EMP3* coding region revealed no mutations in the 132 gliomas investigated. Two single nucleotide polymorphisms were detected in exon 5 in four patients. These sequence variations (SNP-DB rs4893 and SNP-DB rs11671746) are already documented in the SNP database (http://www.ncbi.nlm.nih.gov/SNP/).

Hypermethylation of EMP3. *EMP3* hypermethylation, as defined by a methylation score of 1, 2 or 3 in ≥50% of the investigated CpG sites, was detected in 16 of 22 OII (73%), 28 of 36 OIII (78%), four of five OAII (80%), eight of 11 OAIII (73%), 20 of 24 AII (83%), 21 of 25 AIII (84%), five of 30 pGBM (17%) and eight of nine sGBM (89%) (Figures 1 and 2A). DNA extracted from three non-neoplastic brain samples exhibited no *EMP3* hypermethylation (Figures 1 and 2A).

The extent of *EMP3* hypermethylation, as defined by adding the individual methylation levels (0–3) at each of the 16 investigated CpG sites, was significantly higher in OII, OIII and oligoastrocytomas (OAII and OAIII) with 19q deletions when compared with OII, OIII and oligoastrocytomas without 19q deletions, respectively (Student's *t*-test, OII $P = 0.01$, OIII $P < 0.001$, OAII+OAIII $P = 0.02$). In astrocytic tumors, the extent of *EMP3* hypermethylation was not significantly associated with the allelic status on 19q. The frequency (Fisher's exact test, $P < 0.001$) and extent of *EMP3* (Student's *t*-test, $P < 0.001$) hypermethylation were significantly higher in sGBM (n = 9) when compared with pGBM (n = 30). In fact, most sGBM exhibited methylated CpG sites in the 5′-region of *EMP3* whereas the majority of pGBMs were without any methylated cytosine in this region (Figure 1). Furthermore, three of the five pGBM with *EMP3* hypermethylation carried a combined deletion of 1p and 19q. Upon histological review, each of these three tumors showed areas of oligodendroglial differentiation, thus corresponding to glioblastoma with oligodendroglial component.

EMP3 mRNA expression. Quantitative real-time PCR analysis for *EMP3* mRNA expression was performed in 41 oligodendroglial and 37 astrocytic gliomas (Figure 1). Sixteen of 41 oligodendroglial tumors (39%) but only four of 37 astrocytic tumors (11%) exhibited reduced *EMP3* mRNA levels by at least 50% relative to non-neoplastic brain tissue. The *EMP3* transcript levels were significantly lower in oligodendroglial tumors with allelic losses on 19q when compared with oligodendroglial tumors without 19q losses [mean relative expression level of 0.83 (n = 27) vs. 9.97 (n = 13), $P = 0.01$, Student's *t*-test] (Figure 2B). In contrast, astrocytic tumors did not demonstrate significantly different *EMP3* transcript levels when tumors with and without 19q deletions were compared [mean relative expression level of 38.34 (n = 12) vs. 10.36 (n = 18), $P = 0.08$, Student's *t*-test]. However, *EMP3* mRNA expression was significantly higher in pGBM when compared with either sGBM [mean relative expression level of 60.44 (n = 9) vs. 5.01 (n = 9), $P = 0.008$] or AII [mean relative expression level of 1.21 (n = 9), $P = 0.005$] and AIII [mean relative expression level of 6.60 (n = 10), $P = 0.009$, all Student's *t*-test].

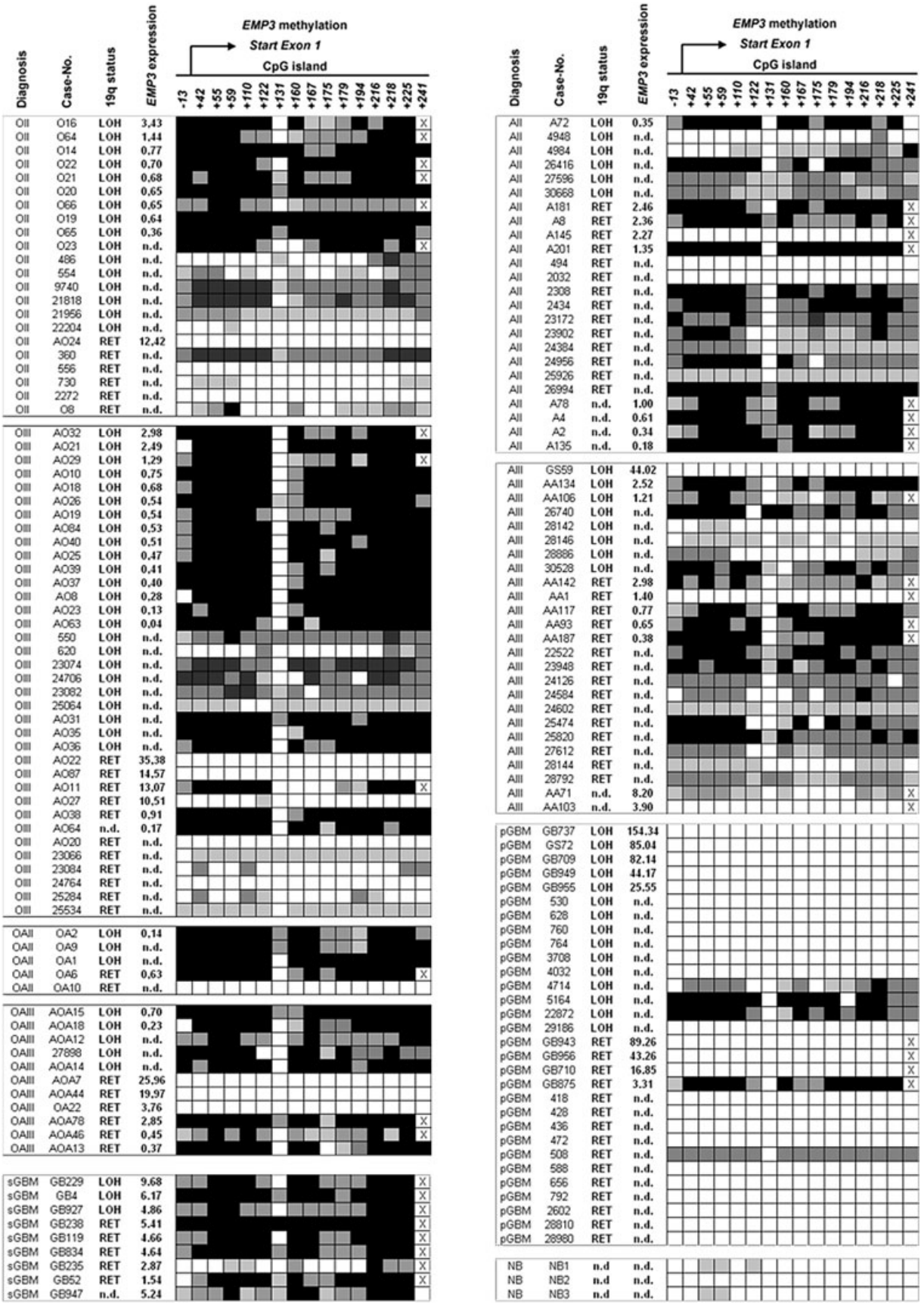

Figure 1. Schematic representation of the results obtained in 162 gliomas and three non-neoplastic brain tissue samples (NB1-3) concerning allelic deletions on 19q, *EMP3* mRNA expression and *EMP3* hypermethylation. Methylation at each of the 16 investigated CpG sites in the *EMP3* promoter region was determined as described in *Materials and Methods*. The location of the transcription start site is indicated on the top of the figure. The results are represented in a 4-tiered semiquantitative grey-scale pattern: *white square*, not methylated (0); *light gray*, weakly methylated (1); gray, moderately methylated (2); *black*, strongly methylated (3). *EMP3* mRNA expression was determined by real-time reverse transcription-polymerase chain reaction analysis and normalized to the mRNA expression of *ARF1*. The *EMP3* expression levels shown are calculated relative to non-neoplastic brain tissue. LOH = loss of heterozygosity; RET = retention of heterozygosity; n.d. = not determined.

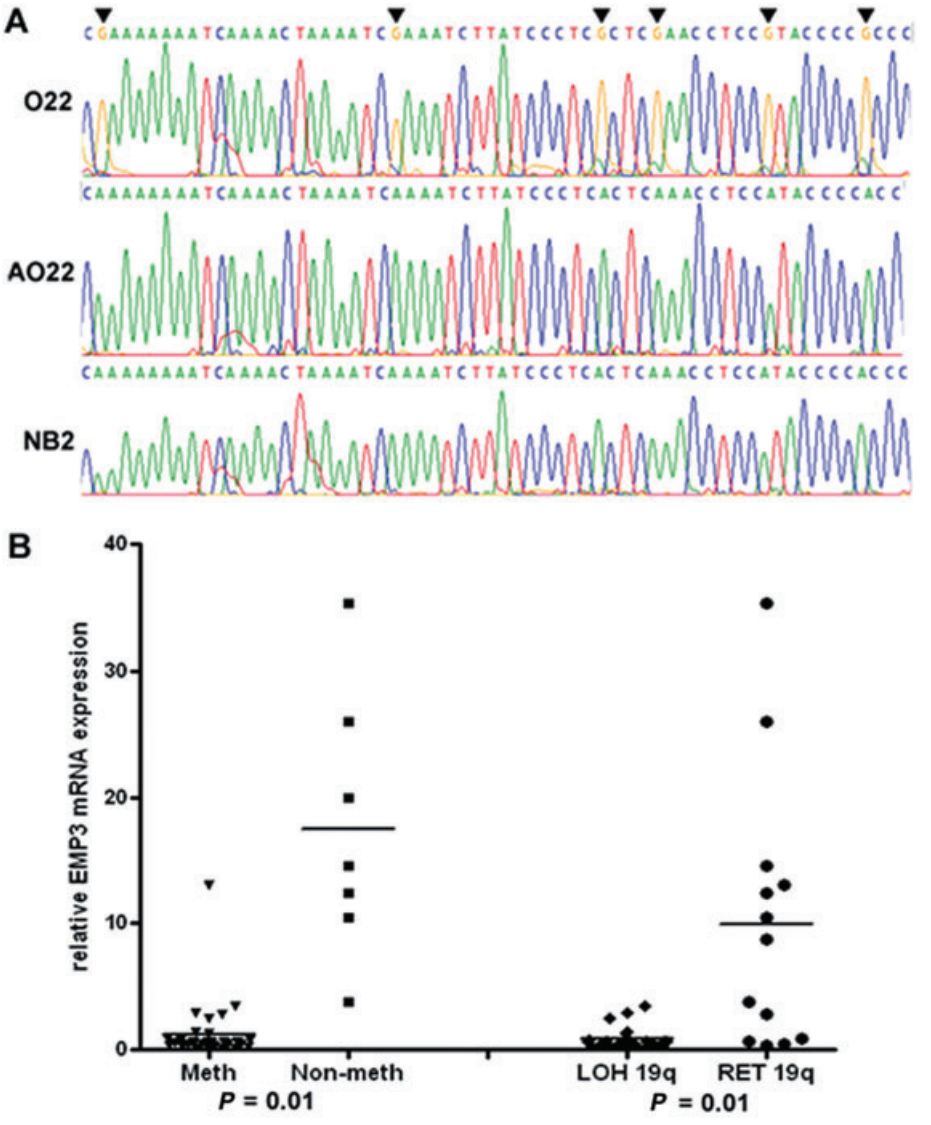

Figure 2. *EMP3 hypermethylation and mRNA expression analysis in oligodendrogliomas and oligoastrocytomas of WHO grade II and III.* **A.** Sequencing of parts of the *EMP3* 5′-CpG-rich region after sodium bisulfite modification revealed methylation of CpG sites in tumor O22 (arrowheads in upper lane) but not in tumor AO22 and in a non-neoplastic brain tissue sample (NB2) (middle and lower lane) (shown is the reverse sequence from chr. 19 nucleotides 53520611 to 53520671, UCSC genome browser, Mar 2006 (hg18) assembly, http://genome.ucsc.edu/). **B.** Dot plot diagrams of *EMP3* expression levels in oligodendroglial tumors with (n = 34) and without (n = 7) *EMP3* hypermethylation (left side; meth., *EMP3* hypermethylated; non-meth., *EMP3* not hypermethylated), as well as with (n = 27) and without (n = 13) allelic losses on 19q (right side; LOH = loss of heterozygosity; RET = retention of heterozygosity). Note significantly lower mean expression levels (indicated by horizontal bars) in tumors with *EMP3* hypermethylation ($P = 0.01$; Student's *t*-test) and tumors with 19q losses ($P = 0.01$).

In oligodendroglial tumors *EMP3* mRNA levels were significantly lower ($P = 0.006$, Student's *t*-test) and more commonly reduced ($P = 0.03$, Fisher's exact test) in tumors with *EMP3* hypermethylation (n = 34) when compared with tumors without *EMP3* hypermethylation (n = 7) (Figure 2B). Similarly, *EMP3* hypermethylation in astrocytic gliomas (25 methylated vs. 12 unmethylated tumors) was significantly associated with lower transcript levels ($P = 0.005$, Student's *t*-test).

Correlation of EMP3 hypermethylation with survival data. To assess the relationship between *EMP3* hypermethylation and patient survival, we investigated 46 patients with oligodendroglial tumors (21 WHO grade II, 25 WHO grade III) and available follow-up data (6). Univariable analysis revealed that *EMP3* hypermethylation was associated with longer overall survival in the entire group of 46 patients ($P = 0.0323$) (Figure 3A). However, only eight tumors (three WHO grade II and five WHO grade III) among the 46 cases lacked *EMP3* methylation. Furthermore, multivariable analysis using Cox's proportional hazards regression model identified 1p/19q loss ($P = 0.04$) but not *EMP3* hypermethylation ($P = 0.81$) as an independent indicator of better prognosis in our patient cohort. In line with this finding, univariable analysis of the 25 patients with WHO grade III tumors revealed no significant survival differences between *EMP3* hypermethylated and unmethylated tumors ($P = 0.44$, Figure 3B). In contrast, significant associations were found between the 1p/19q allelic status and overall survival in the entire group of 46 patients as well as the subgroup of 25 patients with anaplastic tumors (Figure 3C,D).

DISCUSSION

The *EMP3* gene has been suggested as an interesting candidate TSG in gliomas by two independent studies. While Alaminos et al. (1) detected *EMP3* hypermethylation in 39% of the investigated malignant astrocytic gliomas, we found a differential expression of *EMP3* transcripts between gliomas with and without allelic losses on 1p and 19q (32). Here we report on a detailed molecular characterization of *EMP3* aberrations in a large series of 162 astrocytic and oligodendroglial tumors, looking specifically for coding region mutations, DNA hypermethylation and mRNA expression levels. In line with our microarray data (32), quantitative real time-PCR analysis revealed that oligodendroglial tumors with 19q losses had significantly lower mRNA levels when compared with oligodendroglial tumors without 19q losses. Furthermore, we found that *EMP3* hypermethylation is frequent in oligodendroglial tumors and significantly associated with allelic losses on 19q as well as reduced *EMP3* mRNA expression. Taken together, our data indicate biallelic inactivation of *EMP3* by loss of one allele and epigenetic silencing of the other allele in the vast majority of 19q-deleted oligodendroglial tumors, thereby providing support for the hypothesis that *EMP3* is a TSG candidate at 19q13 in

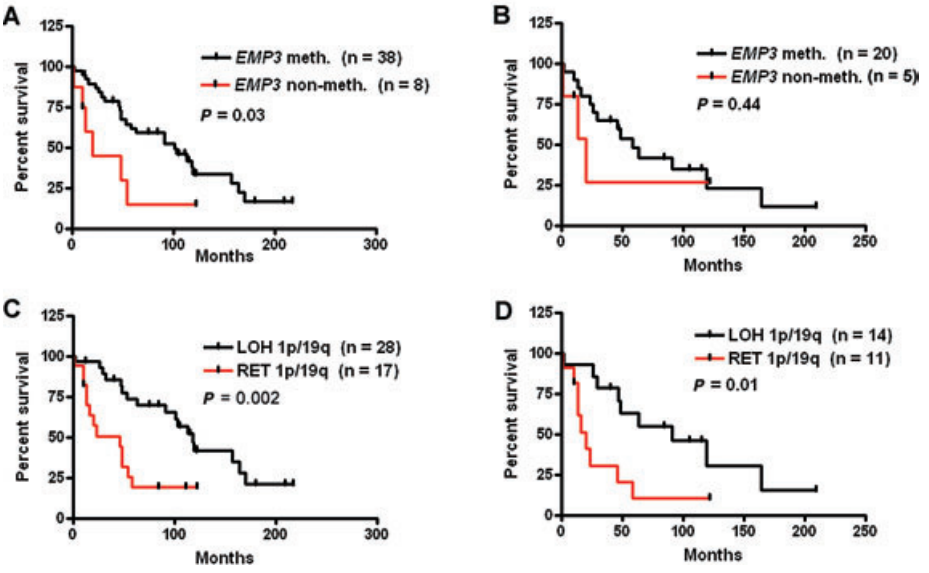

Figure 3. *Univariable analyses of the association between EMP3 hypermethylation (**A,B**) or allelic losses on 1p and 19q (**C,D**) and overall survival in patients with oligodendroglial tumors.* **A,B.** Kaplan–Meier survival curve estimates in relation to the *EMP3* methylation status obtained for 46 patients (**A**), including 21 patients with WHO grade II and 25 patients with WHO grade III tumors, as well as for the subgroup of 25 patients with WHO grade III tumors (**B**). Note association (log rank tests) of *EMP3* hypermethylation with overall survival (OS) in the entire group of oligodendroglial patients (**A**), but not in patients with anaplastic oligodendroglial tumors (**B**). **C,D.** Kaplan–Meier survival curves of the same patient cohort (except for one patient with a WHO grade II tumor) stratified according to the 1p/19q allelic status. Note 1p and 19q losses are significantly associated with longer OS in the entire group of patients (**C**) and in the subgroup of 25 patients with WHO grade III tumors (**D**). meth. = *EMP3* hypermethylated; non-meth. = *EMP3* not hypermethylated LOH = loss of heterozygosity; RET = retention of heterozygosity.

oligodendroglial tumors. However, this conclusion is at variance with a study recently published by Li et al (19). These authors also found frequent *EMP3* hypermethylation in oligodendroglial tumors but did not detect an association between *EMP3* methylation and expression. Nevertheless, they also found 19q losses in the majority of oligodendroglial tumors with low *EMP3* expression while tumors with *EMP3* overexpression had invariably retained both copies of 19q (19).

Our data also show that structural alterations of *EMP3*, in particular tumor-associated mutations in its coding sequence, are rare or absent in both oligodendroglial and astrocytic gliomas. Thus, *EMP3* appears to belong to a growing class of TSG candidates that are preferentially altered by epigenetic mechanisms. Recently identified examples of genes showing frequent epigenetic silencing but rare or absent mutations in gliomas include the *CTMP* (carboxyl-terminal modulator protein) gene at 1q22 in glioblastomas (18), the *CITED4* (CBP/p300-interacting transactivator with glutamic acid/aspartic acid-rich carboxyl-terminal domain 4) gene at 1p34 in oligodendrogliomas with 1p deletion (33), the *RASSF1* (Ras association domain family protein 1) gene at 3p21.3 in gliomas (14), and the protocadherin-gamma subfamily A11 (*PCDHGA11*) gene at 5q31 in astrocytic tumors (41). It is likely that additional TSG candidates that are preferentially down-regulated by hypermethylation will be identified when large-scale epigenetic profiling approaches are applied. In fact, several candidate gene-based studies already reported that multiple genes may be aberrantly hypermethylated in oligodendroglial tumors, including known TSG, such as *CDKN2A*, *CDKN2B*, $p14^{ARF}$ and *RB1*, as well as other genes, such as *DAPK1* (death-associated protein kinase 1), *ESR1* (estrogen receptor 1), *THBS* (thrombospondin 1), *TIMP3* (tissue inhibitor of metalloproteinase 3) and *MGMT* (O(6)-methylguanine-DNA methyltransferase) (2, 4, 21, 42).

Concerning a prognostic role of the *EMP3* promoter status, Alaminos et al (1) reported *EMP3* hypermethylation as an unfavorable prognostic parameter in neuroblastoma patients. In contrast, our finding of a predominance of *EMP3* hypermethylation in oligodendroglial tumors with 19q deletion would suggest a potential association with more favorable prognosis because 19q deletions are frequently combined with 1p deletions, and combined 1p/19q deletions are an independent marker for longer survival in patients with anaplastic oligodendroglial tumors (3, 36). However, while univariable analysis of a series of 46 patients with oligodendroglial tumors of WHO grade II or III revealed a significant association with longer survival for patients with *EMP3* hypermethylated tumors, multivariable analysis did not confirm *EMP3* hypermethylation as an independent prognostic marker and univariable analysis of 25 patients with anaplastic oligodendroglial tumors showed no significant association between *EMP3* hypermethylation and overall survival.

Similar to the findings in oligodendroglial tumors, *EMP3* hypermethylation was frequent in astrocytic neoplasms and was associated with significantly lower *EMP3* transcript levels. However, neither *EMP3* hypermethylation nor *EMP3* mRNA expression showed an association with the 19q deletion status in astrocytic gliomas. Furthermore, *EMP3* mRNA levels were frequently up-regulated in astrocytomas compared with the expression in non-neoplastic brain tissue samples. Thus, it is likely that additional astrocytoma-associated TSG candidates are located on 19q. Our finding of *EMP3* hypermethylation in more than 80% of AII, AIII and sGBM suggests this alteration as a common early event in astrocytoma development rather than a progression-associated change. In fact, *EMP3* hypermethylation is more frequent in diffuse astrocytic gliomas than *TP53* mutation or chromosome 7 gains, which can be detected in approximately 60% and 50% of the cases, respectively (29). Interestingly, our previous studies indicated 19q deletions as a progression-associated aberration in astrocytic tumors, which was more common in AIII than in AII (38–40). The present tumor series showed a similar trend, albeit the percentage of 19q-deleted AII (30%) was higher than previously reported. In any case, it is likely that one or more other TSG are located on 19q that contribute to astrocytoma progression.

A very striking finding of our study is the marked difference in the frequency of *EMP3* hypermethylation between AII, AIII and sGBM (83%–89%) on the one hand and pGBM (17%) on the other hand. This difference would be even more pronounced if those three pGBM with combined deletions on 1p and 19q and oligodendroglial component were removed from the group of pGBM. Then, only two out of 27 pGBM (7%) had *EMP3* hypermethylation. The frequent lack of *EMP3* promoter methylation in pGBM is accompanied by high *EMP3* expression, with mRNA levels being markedly up-regulated in most tumors in relation to the non-neoplastic brain tissue used for reference. The increased expression of *EMP3* transcripts in pGBM as compared with AII, AIII and sGBM is supported by similar data from a recent microarray-based study comparing expression profiles in primary vs. secondary glioblastomas (35). The different frequency of *EMP3* hypermethylation in pGBM vs. sGBM matches well with the concept that sGBM develop by progression from AII or AIII, while pGBM arise *de novo* via distinct molecular pathways (17). In fact, our data suggest the *EMP3* 5′-CpG island methylation status as a possible molecular marker for the distinction between sGBM and pGBM. In line with our data, other authors also reported that methylation frequencies of certain genes, including *HRK* and *TIMP3*, varied between pGBM and sGBM (25, 26).

In conclusion, our data support an important role for *EMP3* hypermethylation as an early epigenetic change in both astrocytic and oligodendroglial tumors. In oligodendroglial neoplasms, *EMP3* hypermethylation was more common in tumors with 19q loss and associated with reduced *EMP3* RNA levels. In astrocytic gliomas, *EMP3* hypermethylation was found in more than 80% of AII, AIII and sGBM, while the vast majority of pGBM demonstrated high *EMP3* mRNA expression in the absence of *EMP3* hypermethylation.

ACKNOWLEDGEMENTS

This study was supported by grants from the Deutsche Krebshilfe to G. R. and A. v. D. (70-3163-Wi3) as well as G. R. and M. S. (70-3088-Sa1), and grants from the German Bundesministerium für Bildung und Forschung (BMBF) within the National Genome Research Network 2 (NGFN-2) to P. L., M. H., G. R., M. W., C. H. and A. v. D. (01GS0460, 01GS0462, 01GR0418 and 01GS0463). B. T. was a scholar of the Studienstiftung des Deutschen Volkes.

REFERENCES

1. Alaminos M, Davalos V, Ropero S, Setien F, Paz MF, Herranz M, Fraga MF, Mora J, Cheung NK, Gerald WL, Esteller M (2005) EMP3, a myelin-related gene located in the critical 19q13.3 region, is epigenetically silenced and exhibits features of a candidate tumor suppressor in glioma and neuroblastoma. *Cancer Res* 65:2565–2571.

2. Alonso ME, Bello MJ, Gonzalez-Gomez P, Arjona D, Lomas J, de Campos JM, Isla A, Sarasa JL, Rey JA (2003) Aberrant promoter methylation of multiple genes in oligodendrogliomas and ependymomas. *Cancer Genet Cytogenet* 144:134–142.

3. Cairncross G, Berkey B, Shaw E, Jenkins R, Scheithauer B, Brachman D, Buckner J, Fink K, Souhami L, Laperierre N, Mehta M, Curran W (2006) Phase III trial of chemotherapy plus radiotherapy compared with radiotherapy alone for pure and mixed anaplastic oligodendroglioma: Intergroup Radiation Therapy Oncology Group Trial 9402. *J Clin Oncol* 24:2707–2714.

4. Dong SM, Pang JC, Poon WS, Hu J, To KF, Chang AR, Ng HK (2001) Concurrent hypermethylation of multiple genes is associated with grade of oligodendroglial tumors. *J Neuropathol Exp Neurol* 60:808–816.

5. Ehrbrecht A, Muller U, Wolter M, Hoischen A, Koch A, Radlwimmer B, Actor B, Mincheva A, Pietsch T, Lichter P, Reifenberger G, Weber RG (2006) Comprehensive genomic analysis of desmoplastic medulloblastomas: identification of novel amplified genes and separate evaluation of the different histological components. *J Pathol* 208:554–563.

6. Felsberg J, Erkwoh A, Sabel MC, Kirsch L, Fimmers R, Blaschke B, Schlegel U, Schramm J, Wiestler OD, Reifenberger G (2004) Oligodendroglial tumors: refinement of candidate regions on chromosome arm 1p and correlation of 1p/19q status with survival. *Brain Pathol* 14:121–130.

7. Griffin CA, Burger P, Morsberger L, Yonescu R, Swierczynski S, Weingart JD, Murphy KM (2006) Identification of der(1;19)(q10;p10) in five oligodendrogliomas suggests mechanism of concurrent 1p and 19q loss. *J Neuropathol Exp Neurol* 65:988–994.

8. Hartmann C, Mueller W, Lass, U, Stockhammer F, von Eckardstein K, Veelken J, Jeuken J, Wick W, von Deimling A (2003) No preferential loss of paternal 19q alleles in oligodendroglial tumors. *Ann Neurol* 54:256–258.

9. Hartmann C, Mueller W, Lass U, Kamel-Reid S, von Deimling A (2004) Molecular genetic analysis of oligodendroglial tumors. *J Neuropathol Exp Neurol* 64:10–14.

10. Hartmann C, Mueller W, von Deimling A (2004) Pathology and molecular genetics of oligodendroglial tumors. *J Mol Med* 82:638–655.

11. Hartmann C, Numann A, Mueller W, Holtkamp N, Simon M., von Deimling A (2004) Fine mapping of chromosome 22q tumor suppressor gene candidate regions in astrocytoma. *Int J Cancer* 108:839–844.

12. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 93:9821–9826.

13. Hong C, Bollen AW, Costello JF (2003) The contribution of genetic and epigenetic mechanisms to gene silencing in oligodendrogliomas. *Cancer Res* 63:7600–7605.

14. Horiguchi K, Tomizawa Y, Tosaka M, Ishiuchi S, Kurihara H, Mori M, Saito N (2003) Epigenetic inactivation of *RASSF1A* candidate tumor suppressor gene at 3p21.3 in brain tumors. *Oncogene* 22:7862–7865.

15. Jenkins RB, Blair H, Ballman KV, Giannini C, Arusell RM, Law M, Flynn H, Passe S, Felten S, Brown PD, Shaw EG, Buckner JC (2006) A t(1;19)(q10;p10) mediates the combined deletions of 1p and 19q and predicts a better prognosis of patients with oligodendroglioma. *Cancer Res* 66:9852–9861.

16. Kleihues P, Cavenee WK (2000) Pathology and genetics of tumors of the nervous system. In: *World Health Organization Classification of Tumours*, 2nd edn. P Kleihues, WK Cavenee (eds), pp. 22–69. IARC Press: Lyon.

17. Kleihues P, Ohgaki H (1999) Primary and secondary glioblastomas: from concept to clinical diagnosis. *Neuro-oncology* 1:44–51.

18. Knobbe CB, Reifenberger J, Blaschke B, Reifenberger G (2004) Hypermethylation and transcriptional downregulation of the carboxyl-terminal modulator protein gene in glioblastomas. *J Natl Cancer Inst* 96:483–486.

19. Li KK, Pang JC, Chung NY, Ng YL, Chan NH, Zhou L, Poon WS, Ng HK (2006) EMP3 overexpression is associated with oligodendroglial tumors retaining chromosome arms 1p and 19q. *Int J Cancer* 120:947–950.

20. Maegawa S, Yoshioka H, Itaba N, Kubota N, Nishihara S, Shirayoshi Y, Nanba E, Oshimura M (2001) Epigenetic silencing of PEG3 gene expression in human glioma cell lines. *Mol Carcinog* 31:1–9.

21. Möllemann M, Wolter M, Felsberg J, Collins VP, Reifenberger G (2005) Frequent promoter hypermethylation and low expression of the MGMT gene in oligodendroglial tumors. *Int J Cancer* 113:379–85.

22. Mora J, Cheung NK, Chen L, Qin J, Gerald W (2001) Loss of heterozygosity at 19q13.3 is associated with locally aggressive neuroblastoma. *Clin Cancer Res* 7:1358–1361.

23. Mueller W, Hartmann C, Hoffmann A, Lanksch W, Kiwit J, Tonn J, Veelken J, Schramm J, Weller M, Wiestler OD, Louis DN, von Deimling A (2002) Genetic signature of oligoastrocytomas correlates with tumor location and denotes distinct molecular subsets. *Am J Pathol* 161:313–319.

24. Nakamura M, Yang F, Fujisawa H, Yonekawa Y, Kleihues P, Ohgaki H (2000) Loss of heterozygosity

on chromosome 19 in secondary glioblastomas. *J Neuropathol Exp Neurol* 59:539–543.

25. Nakamura M, Ishida E, Shimada K, Kishi M, Nakase H, Sakaki T, Konishi N (2005) Frequent LOH on 22q12.3 and TIMP-3 inactivation occur in the progression to secondary glioblastomas. *Lab Invest* 85:165–75.

26. Nakamura M, Ishida E, Shimada K, Nakase H, Sakaki T, Konishi N (2005) Frequent HRK inactivation associated with low apoptotic index in secondary glioblastomas. *Acta Neuropathol (Berl)* 110:402–10.

27. Olek A, Oswald J, Walter J (1996) A modified and improved method for bisulphite based cytosine methylation analysis. *Nucleic Acids Res* 24:5064–5066.

28. R Development Core Team (2007) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. Available at: http://www.R-project.org (accessed April 2007).

29. Reifenberger G, Collins VP (2004) Pathology and molecular genetics of astrocytic gliomas. *J Mol Med* 82:656–670.

30. Reifenberger G, Louis DN (2003) Oligodendroglioma: toward molecular definitions in diagnostic neuro-oncology. *J Neuropathol Exp Neurol* 62:111–126.

31. Taylor V, Suter U (1996) Epithelial membrane protein-2 and epithelial membrane protein-3: two novel members of the peripheral myelin protein 22 gene family. *Gene* 175:115–120.

32. Tews B, Felsberg J, Hartmann C, Hoffmann A, Hahn M, Toedt G, Neben K, Hummerich L, von Deimling A, Reifenberger G, Lichter P (2006) Identification of novel oligodendroglioma-associated candidate tumor suppressor genes in 1p36 and 19q13 using microarray-based expression profiling. *Int J Cancer* 119:792–800.

33. Tews B, Roerig P, Hartmann C, Hahn M, Felsberg J, Blaschke B, Sabel M, Hoffmann A, Toedt G, Neben K, Benner A, von Deimling A, Reifenberger G, Lichter P (2007) Hypermethylation and transcriptional downregulation of the CITED4 gene at 1p34.2 in oligodendroglial tumors with allelic losses on 1p and 19q. *Oncogene* (in press). 19 Feb 2007; [Epub ahead of print].

34. Trouillard O, Aguirre-Cruz L, Hoang-Xuan K, Marie Y, Delattre JY, Sanson M (2004) Parental 19q loss and PEG3 expression in oligodendrogliomas. *Cancer Genet Cytogenet* 151:182–183.

35. Tso CL, Freije WA, Day A, Chen Z, Merriman B, Perlina A, Lee Y, Dia EQ, Yoshimoto K, Mischel PS, Liau LM, Cloughesy TF, Nelson SF (2006) Distinct transcription profiles of primary and secondary glioblastoma subgroups. *Cancer Res* 66:159–167.

36. van den Bent MJ, Carpentier AF, Brandes AA, Sanson M, Taphoorn MJ, Bernsen HJ, Frenay M, Tijssen CC, Grisold W, Sipos L, Haaxma-Reiche H, Kros JM, Van Kouwenhoven MC, Vecht CJ, Allgeier A, Lacombe D, Gorlia T (2006) Adjuvant procarbazine, lomustine, and vincristine improves progression-free survival but not overall survival in newly diagnosed anaplastic oligodendrogliomas and oligoastrocytomas: a randomized European Organisation for Research and Treatment of Cancer phase III trial. *J Clin Oncol* 24:2715–2722.

37. van den Boom J, Wolter M, Kuick R, Misek DE, Youkilis AS, Wechsler DS, Sommer C, Reifenberger G, Hanash SM (2003) Characterization of gene expression profiles associated with glioma progression using oligonucleotide-based microarray analysis and real-time reverse transcription-polymerase chain reaction. *Am J Pathol* 163:1033–1043.

38. von Deimling A, Bender B, Jahnke R, Waha A, Kraus J, Albrecht S, Wellenreuther R, Fassbender F, Nagel J, Menon AG, Louis DN, Lenarzt D, Schramm J, Wiestler OD (1994) Loci associated with malignant progression in astrocytomas: a candidate on chromosome 19q. *Cancer Res* 54:1397–1401.

39. von Deimling A, Nagel J, Bender B, Lenartz D, Schramm J, Louis DN, Wiestler OD (1994) Deletion mapping of chromosome 19 in human gliomas. *Int J Cancer* 57:676–680.

40. von Deimling A, Fimmers R, Schmidt MC, Bender B, Fassbender F, Nagel J, Jahnke R, Kaskel P, Duerr EM, Koopmann J, Maintz D, Steinbeck S, Wick W, Platten M, Muller DJ, Przkora R, Waha A, Blumcke B, Wellenreuther R, Meyer-Puttlitz B, Schmidt O, Mollenhauer J, Poustka A, Stangl AP, Lenartz D, von Ammon K (2000) Comprehensive allelotype and genetic anaysis of 466 human nervous system tumors. *J Neuropathol Exp Neurol* 59:544–558.

41. Waha A, Guntner S, Huang TH, Yan PS, Arslan B, Pietsch T, Wiestler OD, Waha A (2005) Epigenetic silencing of the protocadherin family member PCDH-gamma-A11 in astrocytomas. *Neoplasia* 7:193–199.

42. Wolter M, Reifenberger J, Blaschke B, Ichimura K, Schmidt EE, Collins VP, Reifenberger G (2001) Oligodendroglial tumors frequently demonstrate hypermethylation of the CDKN2A (MTS1, $p16^{INK4a}$), $p14^{ARF}$, and CDKN2B (MTS2, $p15^{INK4b}$) tumor suppressor genes. *J Neuropathol Exp Neurol* 60:1170–1180.