Glioblastoma is the most common primary brain tumor in adults and belongs to the most malignant types of human cancers, as indicated by a median survival time of <1 year in a population-based study (1). The standard treatment for glioblastoma comprises surgical resection followed by local radiotherapy as well as systemic chemotherapy with the DNA alkylating agent temozolomide. According to the results of the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) prospective clinical trial 26981-22981/CE.3, the combination of surgery followed by radiotherapy with concomitant and adjuvant temozolomide increased the median survival of glioblastoma patients by 2.5 months in comparison with treatment with surgery followed by radiotherapy alone (2). Furthermore, the 2-year survival rate increased from 10.4% in the radiotherapy group to 26.5% in the radiotherapy plus temozolomide group (2). Based on molecular analysis of 206 patients from this trial, O6-methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation was shown to play an important role as a molecular predictor for response to temozolomide and longer survival (3). The MGMT gene maps to chromosomal band 10q26 and encodes a DNA repair protein that removes alkyl groups from the O6-position of guanine. Thus, high levels of MGMT may counteract the therapeutic effect of alkylating agents such as temozolomide and lead to treatment failure. On the other hand,
Translational Relevance

This translational study correlates key molecular aberrations with clinical data in primary glioblastoma patients treated by open resection, radiotherapy, and adjuvant chemotherapy with temozolomide. The results confirm MGMT promoter methylation and near-complete resection as independent parameters associated with longer time to progression and overall survival. In contrast, MGMT protein expression and the presence of MGMT sequence polymorphisms were not linked to survival. Furthermore, neither MGMT promoter methylation nor patient outcome was linked to aberrations of the EGFR, TP53, CDK4, MDM2, and PDGFRα genes or losses on chromosomal arms 1p, 10q, and 19q. In summary, our findings provide novel insights into the clinical significance of molecular markers in glioblastomas, which may improve the stratification of glioblastoma patients into prognostically distinct subgroups.

Glioblastomas with MGMT promoter hypermethylation lack expression of this repair enzyme and thereby are more likely to respond to alkylating chemotherapy. Although MGMT promoter methylation has been reported as a predictive biomarker in several independent studies (3–5), the value of a diagnostic assessment of MGMT mRNA or protein expression in glioblastomas is controversial. Some studies have suggested an association between low expression and response to chemotherapy and/or longer survival, but other authors found no relationship between MGMT protein expression and MGMT promoter methylation or survival (for recent review see ref. 6). Furthermore, several studies have reported on associations of MGMT sequence polymorphisms with increased risk for different types of cancer, including gliomas (7), as well as with MGMT promoter methylation in colorectal carcinomas (8). In glioblastoma, however, the role of these polymorphisms with respect to MGMT silencing, response to therapy, and survival is still unclear.

Glioblastomas are characterized by complex genetic and chromosomal aberrations, with the potential relationship of these alterations with MGMT hypermethylation and/or prognosis being largely unknown. In addition, several clinical factors have been established as independent prognostic parameters in glioblastoma patients, including most notably age at diagnosis, clinical status as assessed by the Karnofsky performance score (KPS), extent of resection, and a high score in the Mini-Mental State Examination (9). We report on a single-institution translational study based on 67 primary glioblastoma patients treated by open resection followed by radiation therapy and adjuvant chemotherapy with temozolomide. In this group of patients we determined various molecular parameters, including MGMT promoter methylation, MGMT mRNA and protein expression, presence of MGMT polymorphisms, TP53 mutation, and EGFR, CDK4, MDM2, and PDGFRα amplification, as well as allelic losses on chromosomal arms 1p, 10q, and 19q. The molecular findings were correlated with each other and with various clinical parameters, including data on response to therapy and survival. Our results indicate MGMT promoter hypermethylation and extent of resection as the most important parameters associated with longer survival in glioblastoma patients treated with surgery, radiotherapy, and temozolomide.

Patients and Methods

Patients. A total of 67 adult patients with newly diagnosed, untreated, primary glioblastoma of WHO grade IV were included in this study. The study was not designed as a clinical trial to evaluate the efficacy of different modes of therapy but was primarily intended as a translational investigation to evaluate the prognostic role of various molecular parameters in glioblastoma patients receiving open resection followed by radiotherapy and chemotherapy with temozolomide. All patients were treated between 1998 and 2004 at the Department of Neurosurgery, Heinrich-Heine-University Düsseldorf. Within this period, a total of 306 glioblastoma patients were treated at our institution. Inclusion criteria for the present study were treatment with at least two cycles of chemotherapy with temozolomide as first-line chemotherapy, open resection, sufficient tissue material for molecular analysis, and sufficient follow-up data. From the 306 patients, 196 patients did not receive treatment with temozolomide as first-line chemotherapy or did not qualify for chemotherapy due to clinical conditions and were thus excluded. From the remaining 110 patients, 96 patients received at least two cycles of chemotherapy. Of these, 82 patients were subject to open resection, of whom 13 patients were excluded because of insufficient tumor material and 2 patients were lost to follow-up. Radiotherapy was administered as fractionated focal irradiation at a dose of 2 Gy per fraction given once daily 5 d/wk over a period of 6 wk up to a total dose of 60 Gy. Radiotherapy was followed by adjuvant temozolomide chemotherapy (150-200 mg/m²) according to the standard 5-d schedule every 28 d. A median number of six cycles of temozolomide was administered (range, 2–20 cycles). Therapy was terminated due to tumor progression in 50 patients. Grade 3 or 4 toxicity occurred in 10 patients. Tumor response was evaluated by control cranial magnetic resonance imaging (MRI) initially within 72 h after operation, before initiation of chemotherapy (median time of 7 d before initiation of chemotherapy; SD, 3–21 d), followed by regular MRI at 3-mo intervals until death. End points used in this study were overall survival (OS) and time to progression after initiation of chemotherapy (TTP). Progression was defined according the Macdonald criteria (10). Time to progression after initiation of chemotherapy was chosen to focus on the effect of chemotherapy and to avoid influences due to variations in the time point of initiation of chemotherapy.

The following clinical parameters were determined for each patient: age at the time of the first operation, gender, tumor location, preoperative and postoperative KPS, extent of resection referring to tumor volume in mL as determined in preoperative and early postoperative MRI obtained within 72 h after surgery, time of the last follow-up, and patient status at the last follow-up (alive or deceased). The study was approved by the institutional review board of the Medical Faculty, Heinrich-Heine-University, Düsseldorf (register number 1990). Patients provided written informed consent for their participation in the study. All tumors were histologically classified according to the criteria of the WHO 2000 classification of tumors of the nervous system, which in the case of glioblastoma have been retained in the revised WHO classification of 2007 (11).

Extraction of nucleic acids. Tumor samples obtained during surgery were immediately frozen in liquid nitrogen and stored at −80°C. Extraction of DNA and RNA was carried out by ultracentrifugation as described elsewhere (12). To ensure that the frozen tumor fragments contained a sufficient proportion of tumor cells, each tissue specimen used for nucleic acid extraction was histologically evaluated. From 15 patients without available frozen tumor tissue samples, extraction of DNA was done from paraffin-embedded tissue specimens by using the DNeasy Blood & Tissue Kit (Qiagen). Only tissue samples with
an estimated tumor cell content of 280% were used for the molecular analyses. High-molecular weight DNA was extracted from peripheral blood leukocytes according to a standard protocol.

**MGMT promoter methylation analysis.** The methylation status of the MGMT promoter was determined by methylation-specific PCR as reported before (13). A total of 200 ng of DNA from each tumor was treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Hoffman-La Roche). The primer sequences used to detect methylated MGMT promoter were 5′-gtttgttgattttatggctgac-3′ and 5′-cagcttccaggggaaatctc-3′, which allow for the amplification of a 122 bp fragment. The primer sequences used to detect unmethylated MGMT promoter sequences were 5′-tggttattagttattttggtg-3′ and 5′-ccacaccctcacaataaca-3′, which amplify a 129 bp fragment. The investigated sequences map within the 280-bp fragment that was analyzed in the EORTC/NCIC 26981-22981/CE.3 trial (3). The presence of the respective PCR products was shown by ethidium bromide staining after electrophoresis in 2% agarose gels (Fig. 1). As positive control sample, we used DNA from the A172 glioma cell line (obtained from American Type Culture Collection), which carries a completely methylated MGMT promoter. Genomic DNA extracted from peripheral blood leukocytes served as unmethylated control sample. In addition, a control reaction without any template DNA was done together with each PCR experiment.

**Analysis of MGMT sequence polymorphism.** All patients were screened for the presence of seven distinct MGMT sequence polymorphisms mapping within exons 1, 3, and 5, respectively. The following primer sequences were used: 5′-caggtggttcgtcag-3′ and 5′-gagatgctgctctc-3′ (amplifying a 132-bp fragment from exon 1), 5′-caggcagcagggattacta-3′ and 5′-acaccagcaaggtgattta-3′ (amplifying a 218-bp fragment from exon 3), and 5′-gcgttgcagcctgct-3′ and 5′-tgactgcctggcttct-3′ (amplifying a 226 bp fragment from exon 5). The respective PCR products allowed for sequence analyses of the c.-56C>T variant in exon 1; the c.159C>T (L53L), c.195G>C (W65C), and c.533A>G (K178R) variants in exon 3; and 5′-acaccgcagatggcttagtt-3′ (amplifying a 218-bp fragment from exon 5). Each PCR product was directly sequenced in both directions using cycle sequencing (BigDye cycle sequencing kit, Applied Biosystems) and an ABI PRISM 377 semiautomated DNA sequencer (Applied Biosystems).

**MGMT mRNA expression analysis by real-time reverse transcription-PCR.** Expression of MGMT transcripts was determined by real-time reverse transcription-PCR using the ABI PRISM 5700 sequence detection system (Applied Biosystems). Continuous measurement of the PCR product was enabled by incorporation of SYBR-Green fluorescent dye into the double-stranded PCR products. The transcript level of MGMT was normalized to the transcript level of ARF1 (ADP-ribosylation factor 1, GenBank accession-no. M36340). The respective primer sequences were: MGMT-RT-F, 5′-tcagagatccggatcatgtatg-3′ and MGMT-RT-R, 5′-ggaacagccagcttcgga-3′ resulting in a 102 bp fragment; ARF1-F, 5′-gacagacatcctactacgc-3′ and ARF1-R, 5′-tccacacagaggtttgatg-3′ resulting in a 111 bp fragment. Commercially available adult human brain RNA (BD Biosciences) was used as reference for the mRNA expression analysis.

**Immunohistochemistry for MGMT protein expression.** Of the 67 tumors, 59 were studied by immunohistochemistry on formalin-fixed, paraffin-embedded tissue sections to determine the fraction of MGMT-positive cells. To estimate the variable contribution of microglial cells to the pool of MGMT-positive cells in the tumor sections, each tumor was additionally stained for CD68 expression on consecutive sections. Sections were deparaffinized with xylene for 30 min and rehydrated. The DAKO catalyzed signal amplification (CSA) horseradish peroxidase system and the DAKO Envision system (Dako) were used as the detection systems according to the manufacturer's protocol to show MGMT and CD68 expression, respectively. Immunoreactivity was visualized with 3′,3′-diaminobenzidine as the chromogen. All sections were counterstained with hemalum. For detection of MGMT, we used the mouse monoclonal antibody MT 3.1 (Dako). The mouse monoclonal antibody KP1 (Dako) was used as primary antibody to detect CD68 expression. The primary antibodies were used at final dilutions of 1:500 and 1:1,000 for incubation periods of 20 and 30 min at room temperature, respectively. Negative controls were carried out by omission of the primary antibodies. Each immunohistochemical staining was scored blinded to clinical or molecular information. For the assessment of MGMT protein expression, only nuclear staining was considered. Staining of vascular endothelial cells served as an internal positive control. The fraction of immunopositive tumor cells was evaluated semiquantitatively and categorized according to the following immunoreactivity scores (IRS): 0, no positive tumor cells; 1, <10% positive tumor cells; 2, 10% to 50% positive tumor cells; 3, >50% positive tumor cells (Fig. 2).

**TP53 mutation analysis.** The TP53 gene was screened for mutations in exons 4 to 10 using single-strand conformation polymorphism analysis. The respective exons were amplified by PCR using published primers (14). Each PCR product was screened under different gel electrophoretic conditions with variations in temperature or polyacrylamide gel concentrations. Single-strand conformation polymorphism bands were visualized by silver staining. PCR products with aberrant band pattern were purified and directly sequenced using cycle sequencing and the ABI PRISM 377 DNA sequencer.

**EGFR, CDK4, PDGFRA, and MDM2 gene amplification analyses.** The gene dosage of the EGFR, CDK4, PDGFRA, and MDM2 genes was determined by real-time PCR analysis using the ABI PRISM 5700 (Applied Biosystems) sequence detection system. The amplification level of each gene was normalized to the dosage of the genomic marker D2S1743 (W13306.1) at chromosomal band 2q21.2. The following oligonucleotide primers were used: EGFR, 5′-cagctgcacagctcatc-3′ and 5′-gacgctgctacgagc-3′ (110 bp fragment); CDK4, 5′-gagctgtcctttcctctc-3′ and 5′-ctcctctgtggctc-3′ (102 bp fragment); MDM2, 5′-gtagggccatgaatctgg-3′ and 5′-gaggtcttgctttgctgtg-3′ (158 bp fragment); D2S1743, 5′-tagctgcttgccgtaatg-3′ and 5′-cagggtgcttcagagagct-3′ (131 bp fragment). As reference, we

![Fig. 1. Analysis of different glioblastomas for MGMT promoter methylation using methylation-specific PCR.](Image)

**Table 1.** Analysis of different glioblastomas for MGMT promoter methylation using methylation-specific PCR. Shown are results obtained for three different tumors (GB949, GB911, and GB961). For each tumor, methylation-specific PCR was carried out with DNA either extracted from fresh-frozen tissue samples (cryo) or from formalin-fixed paraffin-embedded tissue samples (paraffin). Note that identical results were obtained with templates from frozen and paraffin-embedded material in each case. Tumors GB949 and GB911 showed PCR products with primers detecting the unmethylated MGMT promoter sequence (M); tumor GB961 showed PCR signals only for the unmethylated MGMT promoter sequence (U). DNA extracted from peripheral blood leukocytes (B) and A172 glioma cells served as negative and positive controls for the methylation-specific PCR analysis of the MGMT promoter.
used DNA extracted from peripheral blood leukocytes or nonneoplastic cerebral tissue samples from two different patients. Positive controls included DNA samples from tumors previously shown by Southern blot analysis to carry amplification of one or more of these protooncogenes (15).

Microsatellite analyses. For loss of heterozygosity (LOH) analysis, we investigated three microsatellite loci each on chromosome arms 1p (D1S211, D1S489, and D1S469), 10q (D10S209, D10S587, and D10S212), and 19q (D19S572, D19S1182, and D19S596). PCR amplification of these microsatellite markers was carried out as reported elsewhere (16). The PCR products were separated by electrophoresis on denaturing 10% polyacrylamide gels and visualized by silver staining. The allele patterns were assessed for allelic imbalance in the tumor DNA as described (16).

Statistical analyses. Clinical and molecular parameters were correlated with survival data established for the 67 glioblastoma patients. TTP was calculated as the interval between onset of the first cycle of temozolomide and the time point of progression on control MRI. OS time was defined as the time between surgery for the primary tumor and death of the patient. Patients alive at the time of their last follow-up were censored. Survival time was estimated by Kaplan-Meier survival curves and compared among patient subsets using log-rank tests. Kaplan-Meier analyses were carried out for the following variables: extent of resection, age at operation, preoperative kps, tumor location, MGMT methylation, MGMT mRNA expression, MGMT immunoreactivity score, TP53 mutation status, and amplification status of CDK4, MDM2 or EGFR; as well as allelic status on 1p, 10q, and 19q. For multivariate analysis of survival time and TTP we used the Cox proportional hazard model. Following a stepwise regression strategy with an inclusion/exclusion \( P \) value of 0.1, a prognostic model was selected from the variables that already had been used in the univariate analysis. All survival and multivariate statistical analyses were carried out with the SAS software (version 8.2). Statistical analyses with Fisher’s exact test were carried out with the GraphPad Prism software (version 4).

Results

Clinical results. The most important clinical data of the investigated patients are summarized in Table 1. The study included 67 patients with newly diagnosed primary glioblastoma of WHO grade IV. The median age at surgery was 56 years (range, 26-80 years). Forty-one patients were males and 26 patients were females. The localizations of the tumor were as follows: temporal \((n = 30)\), parietal \((n = 19)\), frontal \((n = 14)\), and occipital \((n = 4)\). The median preoperative KPS of the patients was 80, with 55 of 67 patients presenting with a preoperative KPS of ≥70. The postoperative KPS was ≥70 in 60 of the 67 patients (89.5%). All patients received at least
two cycles of temozolomide with a median of six cycles being administered. The median tumor volume before surgery was 29.9 mL (range, 0.7-126 mL), whereas the median tumor volume after surgery was 0.6 mL (range, 0.0-85.3 mL). A total of 43 patients received a near-complete resection as defined by a residual tumor volume of <5 mL on early postoperative MRI (Table 1). The follow-up period comprised the period from January 1, 1998, to May 31, 2006, with inclusion of patients in the study until December 31, 2004. Median follow-up was 1,235 days (range, 502-2,919 days). A total of 58 (87%) patients died before database closing and were counted as an event for the end point. Median TTP after onset of chemotherapy in our patients was 157 days (range, 13-621 days) and median OS was 514 days (range, 198-2,002 days; Table 1). These relatively favorable data are likely related to the fact that our study cohort included only patients who were treated with open resection (median tumor volume post surgery, 0.6 mL) and who received at least two cycles of chemotherapy, thus excluding patients with tumor biopsy only and/or too poor clinical condition for chemotherapy.

**MGMT promoter methylation and expression.** Table 2 provides an overview of the major molecular and immunohistochemical findings in our patient cohort. Comparative analysis of the MGMT promoter methylation status by using methylation-specific PCR analysis either with DNA extracted from frozen tumor tissue samples or from formalin-fixed and paraffin-embedded material of 10 different glioblastomas (5 with and 5 without hypermethylation of the MGMT promoter) revealed no differences regarding the source of DNA (Fig. 1). Thus, we assumed that the MGMT promoter methylation data obtained from paraffin-embedded tumor samples (n = 14) were comparable with those obtained from frozen tumor specimens. In total, methylation-specific PCR analysis detected hypermethylation of the MGMT promoter region in 26 of 66 evaluable glioblastomas (39%; Table 2). For individual patients of our study cohort, too small amounts of tumor DNA were available to allow for a comprehensive analysis of all molecular parameters included in this study. This explains why one patient could not be tested for MGMT hypermethylation and why there is some variability in the number of patients investigated for each of the other molecular aberrations.

Expression of MGMT transcripts was investigated by real-time reverse transcription-PCR in the 23 tumors from which sufficient amounts of high-molecular weight RNA could be extracted. Of these 23 tumors, 9 (39%) showed MGMT transcript levels reduced to <50% relative to the nonneoplastic brain tissue reference. The MGMT promoter was hypermethylated in 7 of 9 tumors with reduced MGMT transcript levels and 4 of 14 tumors without reduced MGMT transcript levels (P = 0.036, Fisher's exact test).

Immunohistochemistry for MGMT protein revealed strong expression (IRS 3, >50% positive cells) in 11 tumors, moderate expression (IRS 2, 10-50% positive cells) in 20 tumors, weak expression (IRS 1, <10% positive cells) in 16 tumors, and no expression (IRS 0) in 14 tumors (Fig. 2A-D). When considering the extent of microglial contamination based on CD68 staining of adjacent sections in the assessment of MGMT immunohistochemistry, these numbers changed to 7 tumors with IRS 3, 11 tumors with IRS 2, 20 tumors with IRS 1, and 20 tumors with IRS 0 (Table 2, Fig. 2E and F). The MGMT immunoreactivity scores (after correction for microglial contamination) were compared with the MGMT transcript levels in 21 tumors. MGMT transcript levels were reduced to <50% relative to the nonneoplastic brain tissue reference in 1 of 8 glioblastomas with moderate or strong MGMT immunoreactivity (IRS 2 or 3) and 6 of 13 glioblastomas with weak or absent MGMT immunoreactivity (IRS 1 or 0; P = 0.174, Fisher's exact test). MGMT protein expression (after correction for microglial contamination) was compared with the MGMT promoter methylation status in 57 glioblastomas. The MGMT promoter was methylated in 5 of 17 tumors with IRS 2 or 3 (29%), as well as 19 of 40 tumors with IRS 1 or 0 (48%; P = 0.251, Fisher's exact test).

**MGMT single nucleotide polymorphisms.** The majority of patients carried the wild-type sequence at the seven different single nucleotide polymorphisms (SNP) investigated in the MGMT gene (Table 2). Sequence analysis for the presence of the c.-56C>T SNP within exon 1 done in 61 patients identified 5 patients who were heterozygous and 1 patient who was homozygous for this particular SNP. The MGMT promoter was hypermethylated in 3 of these 6 patients (50%) and 22 of 55 patients (40%) carrying the wild-type sequence at this SNP (P = 0.682, Fisher's exact test). A total of 65 patients were investigated for the c.195G>C (L53L), c.195G>C (W65C), and c.250C>T (L84F) SNPs located in exon 3. Although none of the patients carried the c.195G>C SNP, the c.195G>C and c.250C>T variants were detected in 14 patients (21.5%), including 6 homozygous and 8 heterozygous patients. The presence of these SNPs was not associated with a different MGMT promoter hypermethylation frequency (P = 1.000, Fisher's exact test). TP53 mutations were detected in tumors from 2 of 14 patients carrying the c.250C>T polymorphisms (14%) and 11 of 51 patients without this polymorphism (21.6%; P = 0.717, Fisher's exact test). The c.427A>G (I143V), c.478G>A (G160R),

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**Table 1. Important clinical data of the investigated 67 glioblastoma patients**

<table>
<thead>
<tr>
<th>Summary of clinical data</th>
<th>Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at first surgery in y</td>
<td>56 (26-80)</td>
</tr>
<tr>
<td>Gender, n</td>
<td>41 Male, 26 Female</td>
</tr>
<tr>
<td>Tumor localization, n</td>
<td>14 Frontal, 19 Parietal, 30 Temporal, 4 Occipital</td>
</tr>
<tr>
<td>Karnofsky performance score (preoperative)</td>
<td>80 (20-90)</td>
</tr>
<tr>
<td>Karnofsky performance score (postoperative)</td>
<td>80 (40-90)</td>
</tr>
<tr>
<td>Tumor volume before surgery, mL</td>
<td>29.9 (0.7-126)</td>
</tr>
<tr>
<td>Tumor volume post surgery, mL</td>
<td>0.6 (0-85)</td>
</tr>
<tr>
<td>Cycles of temozolomide</td>
<td>43 (64)</td>
</tr>
<tr>
<td>TTP after onset of chemotherapy, d</td>
<td>157 (13-621)</td>
</tr>
<tr>
<td>OS after diagnosis, d</td>
<td>514 (198-2002)</td>
</tr>
</tbody>
</table>

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and c.533A>G (K178R) SNPs in exon 5 were analyzed in 62 patients. Although the c.478G>A SNP was not detected, three patients showed combined presence of the c.427A>G and c.533A>G SNPs. Again, statistical analysis revealed no association between these SNPs and MGMT promoter hypermethyla-
tion (P = 0.554, Fisher’s exact test). However, all three patients carrying the c.427A>G and c.533A>G SNPshadglioblastomas without EGFR amplification, suggesting an negative association between these SNPs and EGFR amplification (P = 0.016, Fisher’s exact test).

Molecular genetic results obtained for other glioma-associated genes. Mutational analysis revealed TP53 mutations in 13 of 65 investigated glioblastomas (20%). The detected mutations were located in exons 4 (two tumors), 5 (one tumor), 6 (four tumors), 7 (five tumors), and 8 (one tumor), respectively. The identified sequence changes included ten missense mutations, one predicted splice site mutation, one silent mutation, and one small deletion of three base pairs (Supplementary Table S1). None of these mutations were found in the respective patients’ leukocyte DNA, indicating their somatic origin in the tumor cells. PCR-based gene dosage analysis of the EGFR, CDK4, PDGFR, and MDM2 genes identified EGFR gene amplification in 26 of 66 glioblastomas investigated (39%) (Table 2). Amplification of CDK4 was detected in 7 of 64 tumors (11%), MDM2 amplifica-
tion in 5 of 65 tumors (8%), and PDGFR A amplification in 1 of 65 tumors (2%). None of the five tumors with MDM2 amplification carried a TP53 mutation. In line with previous data (1), statistical analyses confirmed TP53 mutation and EGFR amplification as mutually exclusive aberrations in glioblastomas (P = 0.001, Fisher’s exact test). Neither TP53 mutation (P = 1.000) nor EGFR amplification (P = 0.610) was significantly associated with MGMT promoter hypermethylation (Fisher’s exact test).

Allelic losses on 1p, 10q, and 19q. Microsatellite analysis showed allelic losses at one or more of the three markers located on 1p in 4 of 45 tumors investigated (9%; Table 2). Two of these tumors carried partial 1p losses with retention of het-
erozygosity at DIS211 (1p34). LOH at microsatellite markers from 19q was detected in 8 of the 45 tumors (17%). Combined allelic losses on 1p and 19q were restricted to a single case of glioblastoma. Histologic re-evaluation of this particular tumor confirmed the diagnosis of glioblastoma (WHO grade IV) and showed no distinct oligodendroglial tumor component. A total of 31 of 44 glioblastomas studied for allelic losses on 10q (70%) showed LOH at one or more of the three microsatellite loci investigated (Table 2). LOH on 10q was significantly associated with EGFR amplification (P = 0.030, Fisher’s exact test). In contrast, LOH on 10q was associated

<table>
<thead>
<tr>
<th>Molecular parameter</th>
<th>Fraction of cases</th>
<th>Percentage of cases</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGMT hypermethylated</td>
<td>26/66</td>
<td>39%</td>
<td>0.0001</td>
</tr>
<tr>
<td>Low MGMT mRNA expression</td>
<td>9/23</td>
<td>39%</td>
<td>0.023</td>
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<tr>
<td>MGMT protein expression†</td>
<td>≥50%</td>
<td>12%</td>
<td>0.517</td>
</tr>
<tr>
<td>MGMT protein expression†</td>
<td>10-50%</td>
<td>19%</td>
<td></td>
</tr>
<tr>
<td>MGMT protein expression†</td>
<td>&lt;10%</td>
<td>34%</td>
<td></td>
</tr>
<tr>
<td>MGMT c.-56C&gt;T</td>
<td>6/61</td>
<td>10%</td>
<td>0.332</td>
</tr>
<tr>
<td>MGMT c.159C&gt;T</td>
<td>14/65</td>
<td>22%</td>
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<tr>
<td>MGMT c.250C&gt;T</td>
<td>14/65</td>
<td>22%</td>
<td>0.524</td>
</tr>
<tr>
<td>MGMT c.427A&gt;G</td>
<td>3/62</td>
<td>5%</td>
<td>0.684</td>
</tr>
<tr>
<td>MGMT c.478G&gt;A</td>
<td>0/62</td>
<td>0%</td>
<td>n.d.</td>
</tr>
<tr>
<td>MGMT c.533A&gt;G</td>
<td>3/62</td>
<td>5%</td>
<td>0.684</td>
</tr>
<tr>
<td>CDK4 amplification</td>
<td>7/64</td>
<td>11%</td>
<td>0.349</td>
</tr>
<tr>
<td>MDM2 amplification</td>
<td>5/65</td>
<td>8%</td>
<td>0.172</td>
</tr>
<tr>
<td>PDGFR A amplification</td>
<td>1/65</td>
<td>2%</td>
<td>n.d.</td>
</tr>
<tr>
<td>EGFR amplification</td>
<td>26/66</td>
<td>39%</td>
<td>0.522</td>
</tr>
<tr>
<td>TP53 mutation</td>
<td>13/65</td>
<td>20%</td>
<td>0.061</td>
</tr>
<tr>
<td>LOH on 1p</td>
<td>4/45</td>
<td>9%</td>
<td>0.697</td>
</tr>
<tr>
<td>LOH on 19q</td>
<td>8/45</td>
<td>18%</td>
<td>0.187</td>
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<tr>
<td>LOH on 1p and 19q</td>
<td>1/45</td>
<td>2%</td>
<td>0.902</td>
</tr>
<tr>
<td>LOH on 10q</td>
<td>31/44</td>
<td>70%</td>
<td>0.434</td>
</tr>
</tbody>
</table>

Abbreviations: not sig., not significant; n.d., not determined.
*P values as determined by log-rank tests, with P < 0.05 considered as significant.
†Results of immunohistochemical evaluation without correction for microglial contamination.
‡Results of immunohistochemical evaluation after correction for microglial contamination based on CD68 immunostain.

Table 2. Summary of molecular and immunohistochemical results obtained in the investigated glioblastomas and their significance concerning time to progression after initiation of chemotherapy (TTP) and overall survival (OS)
neither with MGMT promoter hypermethylation ($P = 0.742$, Fisher’s exact test) nor with MGMT protein expression ($P = 0.734$, Fisher’s exact test).

### Relationship between molecular findings and clinical data

On univariate analysis, MGMT promoter hypermethylation was significantly associated with longer TTP after initiation of chemotherapy (245 days versus 100 days, $P = 0.0003$) and longer OS (692 days versus 474 days, $P = 0.004$; Table 2 and Supplementary Fig. S1A). In addition, we found that reduced expression of MGMT transcripts associated with longer TTP after initiation of chemotherapy (190 days versus 92 days, $P = 0.023$; Table 2 and Supplementary Fig. S1B). The three patients carrying the c.427A>G and c.533A>G variants in exon 5 showed a significantly shorter OS ($P = 0.045$). In contrast, neither any of the other investigated MGMT SNPs nor the immunohistochemical expression of MGMT protein was associated with TTP after initiation of chemotherapy and OS (Table 2, Supplementary Fig. S1C). Amplification of EGFR, CDK4, MDM2, or PDGFR, and TP53 mutation, as well as allelic losses on 10q or 19q were not significantly associated with patient survival (Table 2). All TP53-mutant tumors were from patients younger than 70 years of age ($n = 61$). However, even when restricting the survival correlation to this age group, as suggested by Batchelor et al. (17), the TP53 mutation status was not associated with TTP ($P = 0.231$) and OS ($P = 0.501$), respectively. EGFR amplification was not associated with distinct outcome in younger patients (<46 years, $n = 13$ patients, 6 patients with EGFR amplification; TTP, $P = 0.889$; OS, $P = 0.310$) or older patients (>46 years, $n = 53$, 20 patients with EGFR amplification; TTP, $P = 0.335$; OS, $P = 0.756$). The four patients with 1p-deleted glioblastomas showed a shorter OS as compared with patients with 1p-intact tumors (447 days versus 531 days, $P = 0.032$). Univariate analysis revealed no significant differences in TTP after initiation of chemotherapy ($P = 0.056$) and OS ($P = 0.492$) when comparing TP53 mutant ($n = 5$) versus TP53 wild-type ($n = 21$) tumors in the group of patients with MGMT-hypermethylated glioblastomas ($n = 26$). In MGMT-unmethylated tumors ($n = 40$; 9 TP53 mutant tumors, 31

### Table 3. Prognostic impact of MGMT methylation status and extent of tumor resection in glioblastoma patients

<table>
<thead>
<tr>
<th>Prognostic impact of MGMT status and extent of resection</th>
<th>OS median (range), d</th>
<th>TTP median (range), d</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGMT meth./unmeth. $n = 26$/$40$</td>
<td>692 (284-1,351)/474 (198-2,002)</td>
<td>245 (63-621)/100 (13-529)</td>
</tr>
<tr>
<td>NCR/noNCR $n = 43$/$15$</td>
<td>600 (198-1,351)/455 (245-1,310)</td>
<td>190 (13-621)/128 (33-357)</td>
</tr>
<tr>
<td>Group 1 MGMT meth./NCR ($n = 18$)</td>
<td>681 (409-1,351)</td>
<td>268 (63-621)</td>
</tr>
<tr>
<td>Group 2 MGMT meth./noNCR ($n = 6$)</td>
<td>499 (284-1,310)</td>
<td>166 (133-351)</td>
</tr>
<tr>
<td>Group 3 MGMT unmeth./NCR ($n = 25$)</td>
<td>507 (198-954)</td>
<td>163 (13-529)</td>
</tr>
<tr>
<td>Group 4 MGMT unmeth./no NCR ($n = 9$)</td>
<td>406 (245-564)</td>
<td>83 (33-159)</td>
</tr>
</tbody>
</table>

Abbreviations: MGMT meth., hypermethylation of the MGMT promoter; MGMT unmeth., no hypermethylation of the MGMT promoter; NCR, near-complete resection.

![Fig. 3. Survival of glioblastoma patients stratified according to MGMT promoter methylation status and extent of resection. The four patient groups correspond to: 1, MGMT-methylated and near-complete resection; 2, MGMT-methylated and incomplete resection; 3, MGMT-unmethylated and near-complete resection; 4, MGMT-unmethylated and incomplete resection. Note that group 1 patients had significantly longer TTP after onset of chemotherapy and longer OS as compared with group 2, 3, and 4 patients. In contrast, group 4 patients had significantly shorter TTP and OS as compared with the other three groups. Group 2 and 3 patients showed similar outcome intermediate to group 1 and group 4 patients, respectively.](https://www.aacrjournals.org/clinica cancerres.aacrjournals.org)
Discussion

We report on a single-institution translational study correlating different molecular features with each other and with clinical data in 67 glioblastoma patients treated by open resection, radiotherapy, and adjuvant chemotherapy with temozolomide. The patients were recruited from 1998 and 2004, i.e., before publication of the EORTC and NCIC trial 26981-22981/CE.3, in which glioblastoma patients treated by radiotherapy combined with concomitant and adjuvant temozolomide showed significantly longer survival as compared with glioblastoma patients treated with radiotherapy alone (2). In line with the translational study based on a subset of patients from this particular trial (3) and several other studies on smaller series of glioblastoma patients (4, 5), our results confirm MGMT promoter hypermethylation as an independent predictive marker of response to therapy (radiotherapy and alkylating chemotherapy), as indicated by significantly longer TTP after initiation of temozolomide treatment, and longer OS. The percentage of MGMT-methylated tumors in our series (39%) is somewhat lower but still comparable with the percentages reported in the EORTC and NCIC trial 26981-22981/CE.3 study (45%; ref. 3) and the study of Eoli et al. (42%, ref. 5), respectively. However, we did not only evaluate our tumor series for MGMT promoter methylation but additionally investigated the clinical significance of MGMT sequence polymorphisms as well as MGMT expression at the mRNA and protein levels. Investigation of the MGMT transcript levels showed significantly lower levels in MGMT-methylated as compared with MGMT-unmethylated tumors. Furthermore, reduced MGMT transcript levels were associated with shorter TTP after onset of chemotherapy. These findings indicate that the MGMT hypermethylation detectable in glioblastomas in situ indeed results in reduced transcriptional activity of the MGMT promoter with consequently lower transcript levels. In contrast, we found that MGMT protein expression was associated neither with the promoter methylation status nor with patient outcome. This result is in line with previous findings from other studies (6, 18) and supports the notion that immunohistochemical assessment of MGMT protein expression does not represent a clinically useful approach (6). The possible reasons that may account for the lack of association of MGMT immunopositivity with lower occurrence of the MGMT promoter methylation and patient survival are not fully understood. Contamination of the tumor tissue by MGMT-positive nonneoplastic elements, such as activated microglial cells, reactive astrocytes, lymphocytic infiltrates, and vascular cells, may cause false positive immunohistochemical results (6, 18). Therefore, we tried to assess the microglial contamination, which may be quite prominent in some glioblastomas, using CD68 immunohistochemistry on consecutive sections. However, even when the extent of microglial contamination was considered in the evaluation of MGMT immunostaining, we still did not detect a significant association of the immunohistochemical results with MGMT promoter methylation and patient survival.

Thus, other molecular mechanisms in addition to promoter methylation likely are involved in the regulation of MGMT protein expression in glioblastomas. For example, p53 has been reported to regulate MGMT expression in minic astrocites and SF767 astrocytomacellswithout affecting promoter methylation (19). A recent study suggested that low expression of mutant p53 was associated with longer survival in glioblastoma patients whose tumors had low MGMT expression (20). In addition, in vitro data indicated that abrogation of p53 wild-type function strongly attenuates temozolomide-induced cytotoxicity in glioma cells, whereas p53 mimetic agents designed to stabilize the wild-type conformation of p53 make glioma cells more susceptible to temozolomide cytotoxicity (21). Nevertheless, we did not detect a significant cooperative effect of MGMT hypermethylation and lack of TTP53 mutation in terms of longer TTP after initiation of chemotherapy and longer OS in our patient cohort. The finding of MGMT protein expression not being significantly different between TP53 mutant and wild-type glioblastomas is in line with a recent study that reported no relationship between MGMT protein expression and p53 immunostaining (22). Thus, our results do not support the hypothesis that low MGMT expression is associated with increased p53 immunopositivity in glioblastomas (23).

The MGMT gene carries several distinct SNPs, such as the c.-56G>T variant in exon 1; the c.159G>T, c.195G>T, and c.250C>T variants in exon 3; and the c.427A>G, c.478G>A, and c.533A>G variants in exon 5. Some of these SNPs have been reported to have an effect on MGMT activity (24). In addition, the c.427A>G and c.533A>G polymorphisms have been associated with increased cancer risk by some authors (25, 26) whereas others did not detect this association (27, 28). In our series, the c.427A>G and c.533A>G SNPs were found in 5% of the patients, i.e., were present at a lower frequency than expected from the reported allele frequency of 11% to 28% in TP53 wild-type tumors), the TP53 mutation status was also not associated with different TTP (P = 0.360) and OS (P = 0.519). Immunohistochemical expression of MGMT did not differ in TP53 mutant (n = 13) versus TP53 wild-type (n = 44) glioblastomas (P = 1.000, Fisher’s exact test).

Univariate analysis for extent of resection confirmed a significantly better clinical course for patients with near-complete resection as compared with patients with residual tumor volume of >5 mL on early postoperative MRI (TTP, 128 days versus resection as compared with patients with residual tumor volume of >5 mL on early postoperative MRI (TTP, 128 days versus 190 days; P = 0.027; OS 455 days versus 600 days; P = 0.017; Table 3 and Supplementary Fig. S1D). In contrast, Kaplan-Meier survival analysis did not reveal significant association of survival with the age at operation (<55 years or ≥55 years), the preoperative KPS (<70 or ≥70), or the tumor location (temporal, parietal, frontal, and occipital).

Based on the finding that extent of resection and MGMT promoter methylation status were both significantly associated with TTP and OS, we divided our patients into the following four groups: group 1, MGMT-methylated and near-complete resection; group 2, MGMT-methylated and >5 mL of residual tumor after resection; group 3, MGMT-unmethylated and near-complete resection; and group 4, MGMT-unmethylated and >5 mL of residual tumor after resection (Fig. 3, Table 3). Univariate analysis revealed that group 1 patients had significantly longer TTP and OS as compared with group 2, 3, and 4 patients. Survival times did not significantly differ between group 2 and group 3 patients, whereas group 4 patients showed significantly shorter TTP and OS as compared with the other three groups. Stepwise forward Cox multivariate regression analysis determined that the best model for predicting OS and TTP after initiation of chemotherapy included MGMT promoter methylation and near-complete resection (Supplementary Table S2).
Caucasians (25). Although we found evidence for a shorter OS in glioblastoma patients carrying the c.533A>G variant, this observation should be interpreted with caution due to the low fraction of patients having this SNP. Recent data on colorectal carcinomas suggested that the c.-56C>T polymorphism is strongly associated with MGMT promoter hypermethylation and loss of MGMT protein expression (8). Our data do not confirm this relationship in glioblastomas. The c.478G>A variant has been associated with resistance to O6-benzylguanine, a pharmacologic inhibitor of MGMT (25). This finding is of potential clinical relevance because treatment with O6-benzylguanine has been used to improve sensitivity of glioblastomas to temozolomide (29). However, we did not detect this particular SNP in any of our patients. Thus, it seems that this variant is rare and its possible role thus restricted to only few glioblastoma patients.

Mutation of the TP53 gene has been found in 60% to 70% of secondary glioblastomas and 25% to 30% of primary glioblastomas (30). The prognostic roles of TP53 mutation and p53 protein expression in glioblastomas are unclear. A population-based study reported that the presence of TP53 mutations was predictive of longer survival on univariate analysis whereas age-adjusted multivariate analysis revealed no difference in survival between patients with and without TP53 mutations (31). Other studies also found no significant association between TP53 mutation or p53 expression and outcome in glioblastoma patients (32, 33). Furthermore, the TP53 mutation rate in glioblastoma patients with long-term survival was similar to that in unselected glioblastoma patients (34). Our study corroborates the lack of prognostic significance of TP53 mutation in primary glioblastoma patients treated by radiotherapy and adjuvant chemotherapy with temozolomide. This finding may at least partly be explained by the fact that glioblastomas without TP53 mutation mostly carry alterations in other p53 pathway genes, such as homozygous deletion of p14ARF or amplification of MDM2 or MDM4 (35). In line with previous data (1), we also found that TP53 mutation was mutually exclusive to EGFR amplification in our glioblastoma patients. Our results do not support an association of TP53 mutation with MGMT hypermethylation in glioblastomas, as it has been suggested for low-grade diffuse astrocytomas (36). Similar to our data, a recent retrospective analysis of 219 glioblastoma patients also found no association between p53 overexpression and MGMT hypermethylation (37).

LOH at polymorphic markers on the long arm of chromosome 10 is the most common genetic alteration in glioblastomas, which is found in 60% to 80% of both primary and secondary glioblastomas (30). In line with these data, we detected allelic losses on 10q in 70% of our patients. Loss of 10q was associated with EGFR amplification but not with promoter methylation of the MGMT gene at 10q26. Similarly, MGMT protein expression did not differ between tumors with and without 10q losses. Thus, the frequent 10q loss probably represents a progression-associated event that is independent from the MGMT methylation status and targets at the inactivation of other tumor suppressor genes on 10q, such as the PTEN gene at 10q23 (38, 39). Data on the prognostic role of 10q deletion in glioblastomas are controversial, with some studies suggesting 10q loss as an indicator of poor outcome whereas others did not report a significant role as prognostic factor (1, 17, 33). In our patients, 10q loss was associated with shorter TTP after onset of chemotherapy nor with reduced OS.

The role of EGFR amplification as a prognostic factor in glioblastomas is debated. Some studies have reported an association of EGFR amplification and/or overexpression with poor prognosis (40, 41). In contrast, other authors could not substantiate a prognostic significance (42, 43), or even reported an association with better prognosis (32, 33). Batchelor et al. (17) suggested an age-dependent prognostic role of EGFR amplification, which was associated with shorter survival in patients younger than 46 years but longer survival in patients older than 46 years of age. We did not find an association of EGFR amplification with survival when analyzing our entire patient cohort and could also not substantiate the reported age-dependent association between EGFR amplification and survival. Amplification of PDGFRA, CDK4, or MDM2 similarly was not linked to survival in our patient cohort. The lack of prognostic relevance of these alterations may be explained by the fact that glioblastomas without these amplifications often carry genetic aberrations in genes encoding other members of the respective signaling pathways (35).

Combined deletion of 1p and 19q is a common feature of oligodendrogial tumors and has been linked to response to radiotherapy and chemotherapy as well as favorable prognosis in patients with anaplastic oligodendroglial tumors (44, 45). In classic glioblastomas, combined 1p/19q losses are rare, but may be more common in so-called glioblastomas with oligodendroglial component (46, 47), which are associated with a better prognosis as compared with classic glioblastomas (48). In our series, only a single tumor showed combined 1p/19q losses. The tumors of three other patients carried 1p deletions in the absence of 19q losses, including two tumors with evidence of partial 1p losses. In total, the OS of these four patients with 1p-deleted glioblastomas was poor, a finding in line with a previous report on unfavorable outcome of patients with glioblastomas carrying partial 1p deletions (49). The biological bases of this association are as yet unclear, but one may speculate that distinct tumor suppressor genes are targeted by the 1p deletions in glioblastomas as compared with oligodendrogliomas.

The importance of extent of resection for progression-free and overall survival of glioblastoma patients has been clearly shown in a prospective randomized phase III trial involving 322 patients (50). Another study based on the EORTC and NCIC trial 26981-22981/CE.3 also revealed extent of tumor resection as prognostically relevant parameter (9). In addition, the MGMT promoter methylation status, younger age, good performance status, and high Mini-Mental State Examination score were identified as independent prognostic factors (9). Our study corroborates MGMT promoter hypermethylation and near-complete tumor resection as favorable markers associated with significantly longer survival in glioblastoma patients treated with temozolomide. Furthermore, our findings suggest an additive effect of both parameters, with those patients having MGMT-methylated and near-complete resection showing the best prognosis. Patients having one of these positive prognostic factors showed an intermediated prognosis, whereas patients having MGMT-unmethylated tumors and incomplete resection showed the worst outcome. These findings stress that complete resection should be attempted in glioblastoma patients, taking advantage of novel resection techniques that facilitate the intraoperative identification of residual tumor tissue (50).

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In summary, our study confirms the role of the MGMT promoter methylation status and the extent of tumor resection as two important factors associated with survival of glioblastoma patients treated with surgery, radiotherapy, and chemotherapy with temozolomide. In contrast, MGMT protein expression, as shown by immunohistochemistry, as well as the molecular detection of MGMT single nucleotide polymorphisms are not linked to the MGMT promoter methylation status and do not provide prognostically relevant information. Similarly, other common glioblastoma-associated genetic alterations, including amplification of EGFR, PDGFRA, CDK4, and MDM2, and mutation of TP53, as well as allelic losses on 1p, 19q, and 19q, were neither linked to the MGMT methylation status nor associated with survival in our series of primary glioblastoma patients.

Disclosure of Potential Conflicts of Interest

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References

associated with resistance to concomitant che-
moradiotherapy in glioblastoma. J Clin Oncol

42. Heimberger AB, Hlatky R, Suki D, et al. Prog-
nostic effect of epidermal growth factor receptor
and EGFRvIII in glioblastoma multiforme patients.

43. Quan AL, Barnett GH, Lee SY, et al. Epidermal
growth factor receptor amplification does not
have prognostic significance in patients with
glioblastoma multiforme. Int J Radiat Oncol Biol

44. van den Bent MJ, Carpentier AF, Brandes AA,
et al. Adjuvant procarbazine, lomustine, and
vincristine improves progression-free survival
but not overall survival in newly diagnosed
anaplastic oligodendroglialomas and oligoastro-
cytomas: a randomized European Organisation
for Research and Treatment of Cancer phase III

trial of chemotherapy plus radiotherapy com-
pared with radiotherapy alone for pure and
mixed anaplastic oligodendroglioma: Intergroup
Radiation Therapy Oncology Group Trial 9402.

46. Kraus JA, Lamszus K, Glesmann N, et al. Mo-
lecular genetic alterations in glioblastomas with
oligodendroglial component. Acta Neuropathol

47. He J, Mokhtari K, Sanson M, et al. Glioblasto-
mas with an oligodendroglial component: a
pathological and molecular study. J Neu-


NOTCH2 positively predicts survival in sub-
groups of human glial brain tumors. PLoS ONE
2007;2:e676.

50. Stummer W, Pichlmeier U, Meinel T, Wiestler
OD, Zanella F, Reulen HJ. Fluorescence-guided
surgery with 5-aminolevulinic acid for resection
of malignant glioma: a randomised controlled
multicentre phase III trial. Lancet Oncol 2006;7:
392–401.
Prognostic Significance of Molecular Markers and Extent of Resection in Primary Glioblastoma Patients

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