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LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Expression of O^6-methylguanine-DNA methyltransferase in childhood medulloblastoma

Denis Faoro · André O. von Bueren · Tarek Shalaby · Davide Sciuscio · Marie-Louise Hürlimann · Lucia Arnold · Nicolas U. Gerber · Johannes Haybaeck · Michel Mittelbronn · Stefan Rutkowski · Monika Hegi · Michael A. Grotzer

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Abstract Medulloblastomas (MB) are the most common malignant brain tumors in childhood. Alkylator-based drugs are effective agents in the treatment of patients with MB. In several tumors, including malignant glioma, elevated O^6-methylguanine-DNA methyltransferase (MGMT) expression levels or lack of MGMT promoter methylation have been found to be associated with resistance to alkylating chemotherapeutic agents such as temozolomide (TMZ). In this study, we examined the MGMT status of MB and central nervous system primitive neuroectodermal tumor (PNET) cells and two large sets of primary MB. In seven MB/PNET cell lines investigated, MGMT promoter methylation was detected only in D425 human MB cells as assayed by the qualitative methylation-specific PCR and the more quantitative pyrosequencing assay. In D425 human MB cells, MGMT mRNA and protein expression was clearly lower when compared with the MGMT expression in the other MB/PNET cell lines. In MB/PNET cells, sensitivity towards TMZ and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) correlated with MGMT methylation and MGMT mRNA expression. Pyrosequencing in 67 primary MB samples revealed a mean percentage of MGMT methylation of 3.7–92% (mean: 13.25%, median: 10.67%). Percentage of MGMT methylation and MGMT mRNA expression as determined by quantitative RT-PCR correlated inversely ($n = 46$; Pearson correlation $r^2 = 0.14$, $P = 0.01$). We then analyzed MGMT mRNA expression in a second set of 47 formalin-fixed paraffin-embedded primary MB samples from clinically well-documented patients treated within the prospective randomized multicenter trial HIT'91. No association was found between MGMT mRNA expression and progression-free or overall survival. Therefore, it is not currently recommended to use MGMT mRNA expression analysis to determine who should receive alkylating agents and who should not.

Keywords Childhood brain tumors · Medulloblastoma · MGMT · Methylation

Denis Faoro and André O. von Bueren contributed equally to this work.

D. Faoro · T. Shalaby · M.-L. Hürlimann · L. Arnold · N. U. Gerber · M. A. Grotzer (✉)
Neuro-Oncology Program, University Children's Hospital, Steinwiesstrasse 75, 8032 Zurich, Switzerland
e-mail: Michael.Grotzer@kispi.uzh.ch

A. O. von Bueren · S. Rutkowski
Department of Pediatric Hematology and Oncology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

D. Sciuscio · M. Hegi
Laboratory of Brain Tumor Biology and Genetics, Department of Neurosurgery, BH-19-110, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland

J. Haybaeck
Institute of Neuropathology, University Hospital of Zurich, Zurich, Switzerland

M. Mittelbronn
Institute of Neurology (Edinger Institute), Goethe-University Frankfurt, Frankfurt/Main, Germany

Introduction

Medulloblastomas (MB) are the most common malignant brain tumors in childhood [1]. They show biological similarities with central nervous system supratentorial primitive neuroectodermal tumors (CNS PNET). Because of the high risk of leptomeningeal dissemination, standard

postoperative treatment includes craniospinal radiotherapy and chemotherapy. Over the last decade, progress has been made in defining clinical and biological prognostic factors that define risk groups and help direct therapy decisions for children with MB. However, our ability to predict response to current treatment is poor.

Alkylating agents, the oldest class of anticancer drugs, are a heterogeneous group of drugs used in the treatment of many cancers including MB [2–4]. Alkylators attacking the O^6 position of guanine fall into two major classes: chloroethylating agents, such as 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1,2-bis(2-chloroethyl)-1-nitrosourea (BCNU), and methylating agents, such as procarbazine and temozolomide (TMZ). At the O^6 position of guanine, induced DNA adducts are repaired by O^6-methylguanine-DNA methyltransferase (MGMT) [5]. This protein acts as a "suicide" acceptor protein for the alkyl group, restoring DNA to normal but inactivating itself in the process [6, 7]. Thus, the ability of a cell to withstand such damage appears to be directly related to the number of MGMT molecules it contains and to the rate of de novo synthesis of MGMT [8]. As such, it is a unique DNA repair protein. There are no back-up mechanisms or any redundancy in the DNA repair process of O^6-alkylguanine lesions [9]. If repair of the alkylation does not occur, most O^6-chloroethylguanine lesions are converted to G–C interstrand crosslinks within 8–12 h [10]. These are very poorly repaired in mammalian cells, forcing a halt to the DNA replication, formation of single- and double-strand breaks, and induction of p53 and p21, which leads to apoptotic and necrotic cell death [6]. O^6-methylguanine can pair with thymine during DNA replication, resulting in conversion of guanine-cytosine to adenine–thymine pairs in DNA [11]. However, the most likely explanation for the cytotoxicity of O^6-methylguanine lesions is that it emanates from the induction of mismatch repair at O^6-mG:T sites [12]. Because mismatch repair targets the newly synthesized strand for re-synthesis, O^6-mG on the parent strand remains and repetitive cycling of aberrant repair takes place, which induces apoptosis in a fairly efficient manner [6].

Several studies have suggested a favorable association between *MGMT* methylation/low MGMT levels and response to chloroethylating and methylating agents in adults and children with malignant glioma [13–16]. In pediatric MB, He et al. [17] demonstrated absence of MGMT mRNA and protein expression in D425 Med and D458 Med cell lines. Hongeng et al. [18] found higher levels of MGMT in MB when compared with gliomas. A single MB examined was hypermethylated at *MGMT* [8]. Rood et al. [19] found methylation of *MGMT* in 28 of 37 MB samples. However, no correlation was found between *MGMT* methylation and mRNA expression. Microarray-based screening in 35 MB revealed that high levels of MGMT correlated with unfavorable survival outcome [20]. Bobola et al. [21] studied MB cell lines and found that MGMT is a major determinant of BCNU and TMZ sensitivity.

In this study, we investigated *MGMT* methylation in MB/PNET cell lines and primary MB and investigated whether *MGMT* methylation reflects the expression of the gene. We then investigated the response of MB/PNET cells to TMZ and CCNU, and evaluated the potential prognostic significance of MGMT expression in a relatively uniformly treated patient population.

Materials and methods

Cell lines

DAOY human MB cells were purchased from the American Type Culture Collection (Rockville, MD). SW48 (colon carcinoma), D341 (MB), D425 (MB), UW228-2 (MB), Med-1 (MB), PFSK (PNET), and PNET-5 cells were gifts from several universities and medical centers. DAOY, D341, and D425 cells were cultured in Richter's Zinc Option medium/10% fetal bovine serum (FBS); (D341 and D425 were additionally cultured with 1% nonessential amino acids); PFSK cells were cultured in RPMI 1640/10% FBS; and SW48, PNET-5, Med-1, and UW228-2 cells were cultured in Dulbecco's modified Eagle's medium (Gibco Invitrogen, Basel, Switzerland)/10% FBS. All cell cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere.

Peripheral blood lymphocytes

Peripheral blood lymphocytes (PBL) cells were separated from the first author's blood using Leucosep (Greiner Bio-One, Frickenhausen, Germany) according to the manufacturer's instructions.

Drugs and chemicals

Stock solutions of TMZ (Schering-Plough, Kenilworth, NJ, USA) and O^6-benzylguanine (Sigma-Aldrich, Basel, Switzerland) were prepared in DMSO and stored at −80°C. CCNU (Lomustine; Bristol-Myers Squibb, Baar, Switzerland) was prepared fresh in 10% ethanol (0.05 mg per ml).

Quantitative RT-PCR

Total RNA isolation, reverse transcription reactions, and RT-PCR were performed as described previously [22]. Kinetic real-time PCR quantification of target genes was

performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland), as described previously [23]. Primers and probes for *MGMT* (assay ID: Hs01037698_m1) and the endogenous control 18S rRNA (assay ID: Hs99999901_s1) were purchased from Applied Biosystems. Experiments were performed in triplicate for each data point. Relative mRNA expression of target genes was calculated by using the comparative C_T method [24]. The amount of MGMT was normalized to 18S rRNA and calibrated to a commercially available normal human cerebellum probe (Clontech, Saint-Germain-en-Laye, France).

Western blot analysis

The expression of MGMT and β-actin protein was assessed by western blot analysis. In brief, pellets of MB/PNET cells were lysed with lysis buffer (1 ml/10^7 cells, 50 mM Tris–HCl buffer pH 8.0, 150 mM NaCl, 1% Nonidet P40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM EDTA, and 1 mM EGTA pH 8.0) containing fresh protease inhibitors (Complete; Roche, Basel, Switzerland) and incubated on ice for 30 min. After estimating the protein concentration by the BCA method (Pierce, Rockford, USA), 30 µg total protein lysates were separated by 10% SDS polyacrylamide gels and the gels were subjected to immunoblotting. Nonspecific binding sites were blocked by 3 h incubation in TBST (10 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20) supplemented with 5% nonfat milk powder. Membranes were incubated overnight at 4°C with a 1:200 dilution of mouse monoclonal primary MGMT antibody (Lab Vision, Wohlen, Switzerland). Membranes were then washed three times at room temperature in TBST for 30 min each time, and bound Ig was detected using anti-isotype monoclonal secondary antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany). The signal was visualized by enhanced chemiluminescence ECL (Amersham Biosciences, Dübendorf, Switzerland) and autoradiography. Then immunoblotting with a 1:5,000 dilution of a mouse monoclonal primary β-actin antibody (Sigma-Aldrich, Buchs, Switzerland) was performed to verify equivalent amounts of loaded protein.

DNA and RNA isolation from formalin-fixed and paraffin-embedded tumor samples

The Recover All Total Nucleic Acid Isolation kit (Ambion, Austin, USA) was used to isolate DNA and RNA from archive MB samples. Briefly, 5-µm sections were cut from 10% neutral-buffered formalin-fixed (24 h), paraffin-embedded blocks, placed in RNase-free, 1.5-ml Eppendorf tubes and deparaffinized with xylene. The samples were washed with ethanol, centrifuged for 5 min at 13,000*g*, air-dried, and resuspended in 400 µl digestion buffer (Ambion) containing 4 µl proteinase K (60 units/µl). Samples were vortexed and incubated for 3 h at 50°C, centrifuged at 1,400*g* to extract RNA and incubated for 24 h at 50°C, then centrifuged at 1,400*g* to extract DNA. After complete digestion, 480 µl Isolation Additive was added and the samples were vortexed. Then, 1.1 ml 96% ethanol was added to each sample and mixed in carefully. The sample/ethanol mixture was applied to a filter cartridge and centrifuged for 30 s at 10,000*g*. After the filter cartridge was washed and the residual fluid was removed, 60 µl of DNase mix were added for RNA extraction or 60 µl of RNase for DNA extraction to the center of the filter. DNase was incubated for 30 min at room temperature and the RNase was incubated for 30 min at 37°C. The nucleic acid was eluted with 30 µl of nuclease-free water heated to 95°C. DNA from cell lines and PBL was isolated using the QIAamp DNA Mini Kit (Qiagen, Switzerland) according the manufacturer's protocol [25].

Methylation-specific polymerase chain reaction

Methylation-specific PCR (MSP) was performed as previously described [26]. In brief, genomic DNA was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research, Orange, USA). The reaction transforms unmethylated cytosine residues into uracil but not their methylated counterpart [19]. Purified, bisulfite-treated DNA was subjected to MSP using a two-step approach with nested primers. The first round of PCR amplifies both the methylated and unmethylated sequence of *MGMT* that serves as template in the second step in which two independent reactions are performed amplifying either the methylated or unmethylated *MGMT* sequence using methylation-specific primers. DNA from SW48 cells served as positive control and PBL as negative control. The PCR products were separated and visualized on a 4% agarose gel.

Pyrosequencing

The pyrosequencing experiments were performed by EpigenDX (Worcester, USA). This pyrosequence assay analyzed MGMT promoter methylation in eight CPG positions (CPG8–CPG15) extending from nucleotides +17 to +81 relative to transcription started site (based on Ensembl sequence: ENST00000306010) [27, 28]. A standard pyrosequencing sample preparation protocol was applied as described previously [27, 29]. Briefly, PCR was performed using the appropriate primers with a final volume of 50 µl. The initial denaturation (97°C, 15 min) was followed by 38 cycles of 1 min at 95°C, 1 min at 47.5°C, 1 min at 72°C, and a final extension step at 72°C for

10 min. We subjected 40 µl of the PCR product to pyrosequencing. The sequencing reaction was performed on an automated PSQ 96MA System (Biotage, Uppsala, Sweden) using the Pyro Gold reagents kit (Biotage). Purification and subsequent processing of the biotinylated single strand DNA was performed according to the manufacturer's instructions. Resulting data were analyzed and quantified with the PSQ 96MA 2.1 software (Biotage). Each sample was analyzed in triplicate by individual PCR reactions using the same bisulfite preparation as template. Methylation values $\leq$5% were considered as potential background noise signals of questionable significance, in concordance with other publications [30–32].

Cell proliferation

The appropriate number of MB cells were seeded in 96-well plates, treated the next day with various concentrations of TMZ or CCNU, and incubated for 5 and 4 days, respectively, at 37°C in a humidified 5% CO_2 atmosphere. The specific MGMT inhibitor, O^6-benzylguanine, was added 2 h before TMZ or CCNU treatment at a concentration of 25 µM [33]. A colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay (Promega, Wallisellen, Switzerland) was used to quantitate cell viability as previously described [22, 34]. Following incubation, 10 µl of MTS working solution was added to each culture well, and the cultures were incubated for 3.5 h at 37°C in a humidified 5% CO_2 atmosphere. Each experiment was performed in triplicate. The absorbance values of each well were measured with a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 490 nm. IC50 values were calculated from the regression curve as previously described [35]. All proliferation assays were repeated as independent experiments at least twice.

Patients and therapy

Tumor material was available from two sets of archival MB samples. One set contained tumor samples from patients diagnosed in Zurich, Switzerland ($n = 57$) and Cairo, Egypt ($n = 10$). All these samples were reviewed by two neuropathologists (J.H., M.M.) and diagnosed as MB. However, the clinical information (mainly metastatic stage) for this set is incomplete and the therapy received was not homogeneous. For the other set, tumor material was available from 47 children registered between December 1991 and October 1997 to the prospective randomized multicenter trial HIT'91. After surgery, patients were randomized to receive either craniospinal radiotherapy followed by maintenance chemotherapy ('maintenance arm') or neoadjuvant chemotherapy and craniospinal radiotherapy ('sandwich arm') as described [36]. Briefly, neoadjuvant 'sandwich' chemotherapy consisted of procarbazine followed by two cycles of ifosfamide/etoposide, high-dose methotrexate, and cisplatin/cytarabine; poor responders received eight additional cycles of carboplatin/CCNU/vincristine after radiotherapy. Maintenance chemotherapy consisted of eight courses of cisplatin, CCNU, and vincristine. Radiotherapy consisted of 35.2 Gy to the neuraxis, a boost of 20 Gy to the posterior fossa, and an additional boost to macroscopic metastases if present.

Statistical analysis

All data are expressed as mean ± SD. GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA) software was used to calculate IC50 values and to determine correlations between IC50 values of TMZ or CCNU and MGMT mRNA expression. Survival functions were estimated using the method of Kaplan and Meier, and the log-rank test was used for comparison. Progression-free survival (PFS) was defined as time from the date of diagnosis to the date of first progression, relapse, or death from any cause, or to the date of the last contact. Overall survival (OS) was defined as the time from the date of diagnosis to death from any cause or to last contact. Standard errors are expressed as plus-minus values. The univariable analyses were done on a local significance level of 5% and were not adjusted for multiple comparisons. Multivariable Cox regression analysis was used to analyze possible confounding variables to the influence of MGMT status on the risk of recurrence or death. The forward stepwise model selection procedure according to Collet was used to define the final model (P value of likelihood-ratio test $\leq$0.05 as inclusion criteria; likelihood-ratio test $\geq$0.10 as exclusion criteria). The following variables were entered into the analyses: gender, age at diagnosis, histologic subtype (classic, desmoplastic, and anaplastic medulloblastoma), stage [no metastases, no macroscopic metastasis without cerebrospinal fluid (CSF) examination, microscopic without macroscopic, and macroscopic metastases], extent of primary tumor resection (gross total resection, incomplete resection), and treatment regimen ('maintenance arm', 'sandwich arm'). Approval to perform the study was obtained from the Institutional Review Board.

Results

MGMT status of MB/PNET cells

To measure the methylation status of *MGMT* promoter in MB/PNET cells, we performed two different assays, MSP and the more quantitative pyrosequencing assay. MSP

demonstrated *MGMT* promoter methylation in one (D425) of seven MB/PNET cell lines tested (Fig. 1a). This result was validated by pyrosequencing. The mean percentage of *MGMT* methylation in D425 cells was 81.9% compared with 1.7–3.0% in the other six MB/PNET cell lines tested (Fig. 1b). In D425 human MB cells where *MGMT* promoter methylation was demonstrated, *MGMT* mRNA expression as determined by RT-PCR and MGMT protein expression as determined by western blotting were nearly absent (Fig. 1c, d).

MB/PNET cell sensitivity to TMZ and CCNU

The cytotoxic effects of TMZ and CCNU were examined in seven MB/PNET cell lines by using the MTS assay. The IC_{50} values (averaged from two independent experiments) for TMZ ranged from 3.9 to 340.1 μM (D425: 3.9 μM; PFSK: 78.9 μM; DAOY: 163.5 μM; D341: 234.6 μM; UW-228-2: 267.2 μM; Med-1: 340.1 μM; and PNET-5: 227.7 μM) (Fig. 2a). In the case of CCNU, the IC_{50} values ranged from 4.1 to 100.7 μM (D425: 4.1 μM; PFSK: 27.1 μM; DAOY: 43.9 μM; D341: 31.0 μM; UW-228-2: 73.2 μM; Med-1: 100.7 μM; and PNET-5: 34.4 μM) (Fig. 2b). To further define the role of MGMT in mediating resistance to alkylating agents in MB/PNET, we tested whether the MGMT inhibitor O^6-benzylguanine is able to sensitize to TMZ and CCNU [4, 7]. O^6-benzylguanine co-exposure had no effect in D425, little effect in PFSK and D341, but shifted the IC_{50} in the MTS assay from 154.7 to 24.0 μM in DAOY, from 293.5 to 53.9 μM in UW-228-2, from 315.7 to 21.5 μM in Med-1, and from 225 to 13 μM in PNET-5 (Fig. 2a). Sensitization to CCNU-mediated cell killing by O^6-benzylguanine co-exposure was similar. We observed no effect in D425, little effect in PFSK and D341, but O^6-benzylguanine shifted the IC_{50} in the MTS assay from 33 to 12.3 μM in DAOY, from 69.3 to 33.2 μM in UW-228-2, from 83 to 9.6 μM in Med-1, and from 36.3 to 29 μM in PNET-5 (Fig. 2b).

We then compared sensitivity towards TMZ and CCNU with mRNA expression of MGMT. IC_{50} values—determined

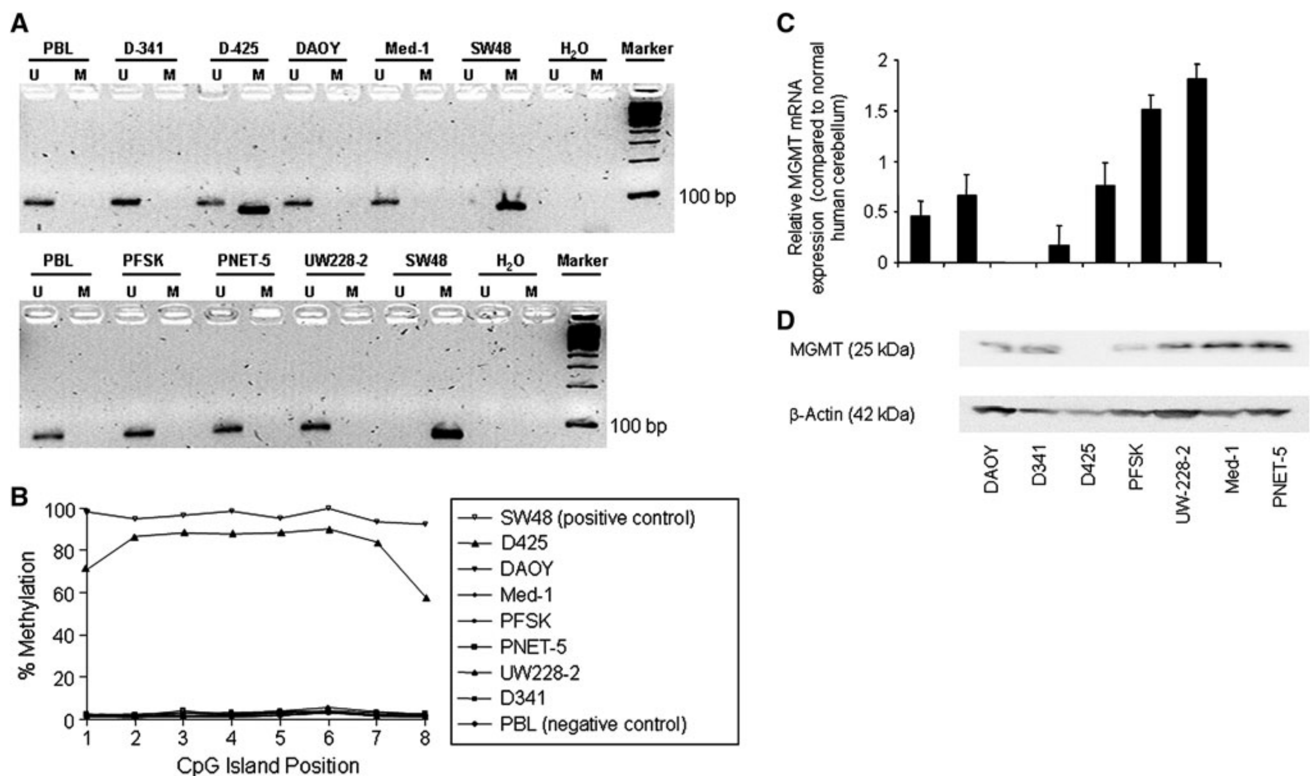
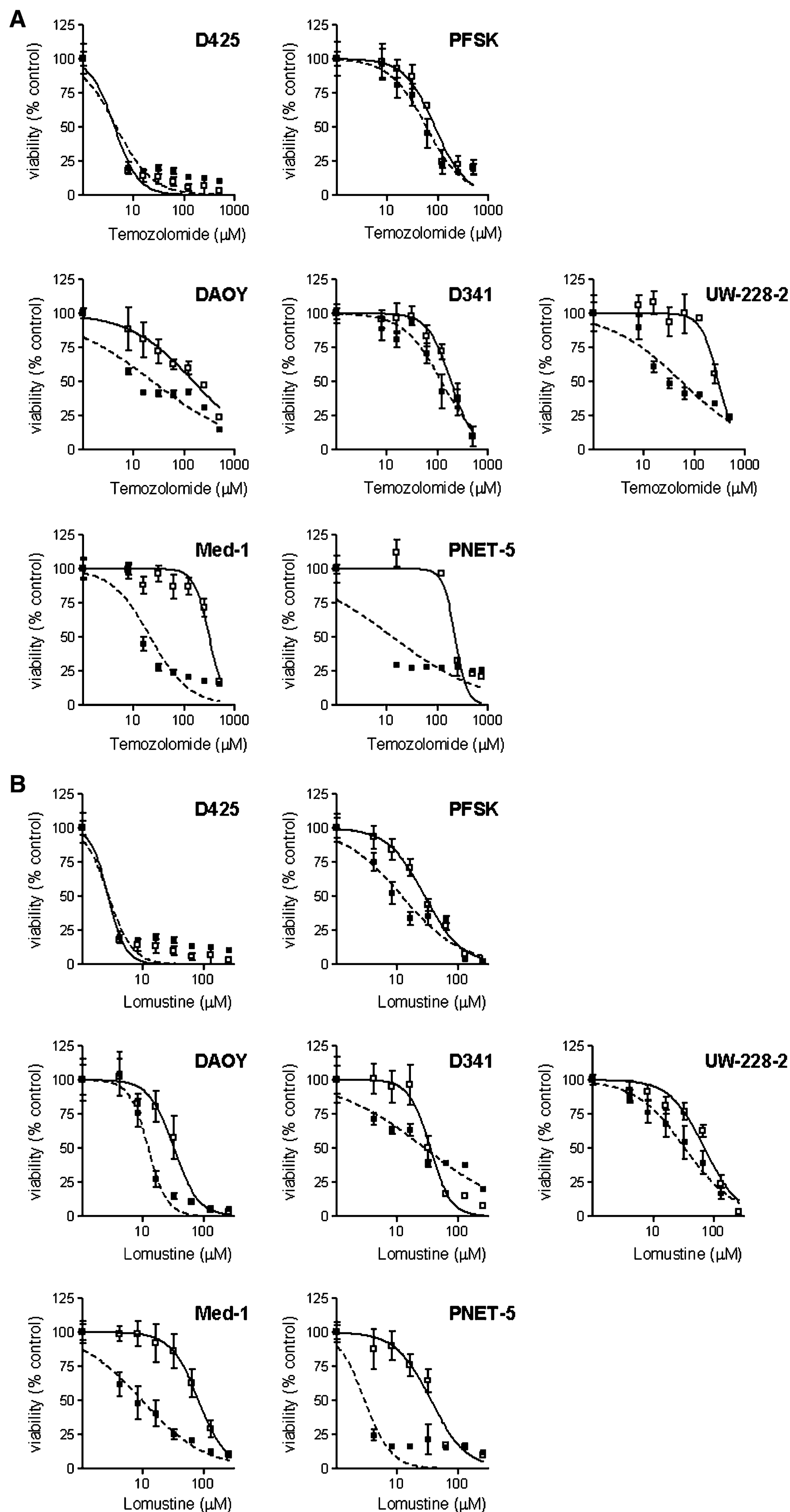


Fig. 1 Methylation status of the *MGMT* promoter in MB/PNET and control cells, as determined by nested methylation-specific PCR (**a**) and pyrosequencing (**b**). Controls included peripheral blood lymphocytes (*PBL*) with unmethylated (*U*) *MGMT* promoter, SW480 colon cancer cells with methylated (*M*) *MGMT* promoter, and water. A 100-bp marker ladder was loaded to estimate molecular size. D425 contains a methylated *MGMT* promoter, whereas all other MB/PNET cells harbor only an unmethylated promoter. Pyrosequencing assay measured level of methylation in eight CpG island of *MGMT* promoter regions (*x*-axis). The *y*-axis shows the percentage of methylation in each CpG-island. **c** MGMT mRNA as determined by quantitative RT-PCR. Values represent the relative MGMT mRNA expression compared with a normal human cerebellum sample ($n = 3$; ±SD). **d** MGMT protein expression as determined by western blot analysis

Fig. 2 TMZ- (**a**) and CCNU-mediated (**b**) cytotoxicity in human MB/PNET cells as determined by the MTS assay. Experiments were performed with (*black squares*) or without (solvent only) O^6-benzylguanine pre-treatment (25 μM; 2 h). Values represent the mean percentage of viability (representative from two independent experiments; $n = 5$; ±SD). **c** Comparison of TMZ sensitivity and MGMT mRNA expression in MB/PNET cells. **d** Comparison of CCNU sensitivity and MGMT mRNA expression in MB/PNET cells



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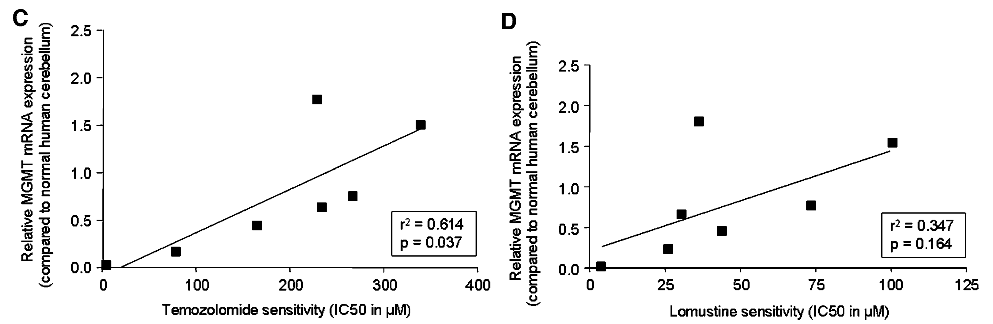

in the acute growth inhibition assay—of TMZ and MGMT mRNA expression levels correlated significantly (Fig. 2c; $r^2 = 0.61, P = 0.037$). In the case of CCNU, the correlation was not significant (Fig. 2d; $r^2 = 0.33, P = 0.18$).

MGMT promoter methylation and *MGMT* mRNA expression in primary MB samples

We then used the pyrosequencing method to measure *MGMT* promoter methylation in DNA extracted from 67 formalin-fixed, paraffin-embedded primary MB samples. Pyrosequencing revealed a mean percentage of *MGMT* methylation of 3.7–92% (mean: 13.25%, median: 10.67%) (Fig. 3a). Quantitative RT-PCR of 46 corresponding MB samples with sufficient tumor material to perform RT-PCR revealed MGMT mRNA expression levels of 0.00–6.68 (mean: 1.67, median: 1.01; relative to normal cerebellum). Mean percentage of *MGMT* methylation correlated inversely with *MGMT* mRNA expression (Pearson correlation $r^2 = 0.14, P = 0.01$; Fig. 3b). Since the clinical information for this set of primary MB samples was incomplete and the therapy received not homogeneous, a survival analysis was not performed. We then used another set of primary MB samples to correlate *MGMT* status and survival outcome. For this second set, clinical information was complete, with cDNA already available from a previous study [37], but with no genomic DNA available to perform MGMT methylation analysis. We therefore used quantitative RT-PCR instead of pyrosequencing.

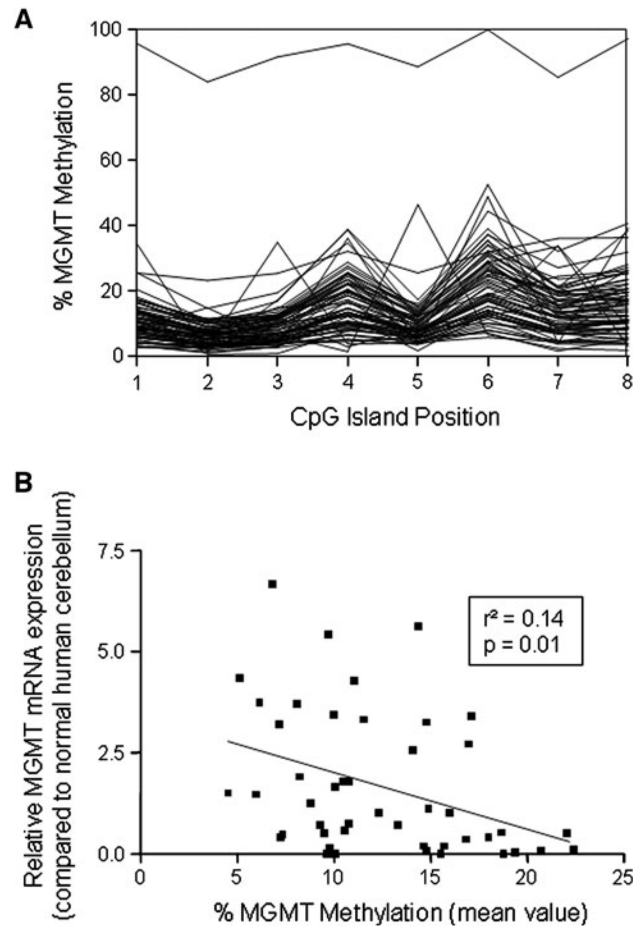

Fig. 3 **a** *MGMT* promoter methylation in 67 formalin-fixed paraffin-embedded primary MB samples as determined by pyrosequencing. **b** Mean percentage of *MGMT* promoter methylation correlates with the MGMT mRNA expression levels as determined by quantitative RT-PCR ($n = 46$)

MGMT mRNA expression and survival outcome in primary MB samples

In an attempt to ascertain whether MGMT mRNA expression predicts survival outcome probability, we analyzed MGMT mRNA expression in formalin-fixed, paraffin-embedded tumor samples from well-documented patients treated within the prospective randomized multicenter trial HIT'91. Samples from 47 patients were available for analysis. Median age at diagnosis was 7.5 years (range, 2.9–17.0). Twenty-eight patients were male, 19 were female. Twenty-three patients had no metastases, 3 patients had no macroscopic metastases and no CSF examination at diagnosis, 13 patients had microscopic CSF but no macroscopic metastases, and 8 patients had macroscopic central nervous system metastases. Histologic subtype was classic in 42, desmoplastic in 3, and anaplastic in 2 patients. Thirty-two patients had no post-operative macroscopic primary residual tumor as assessed by early-postoperative

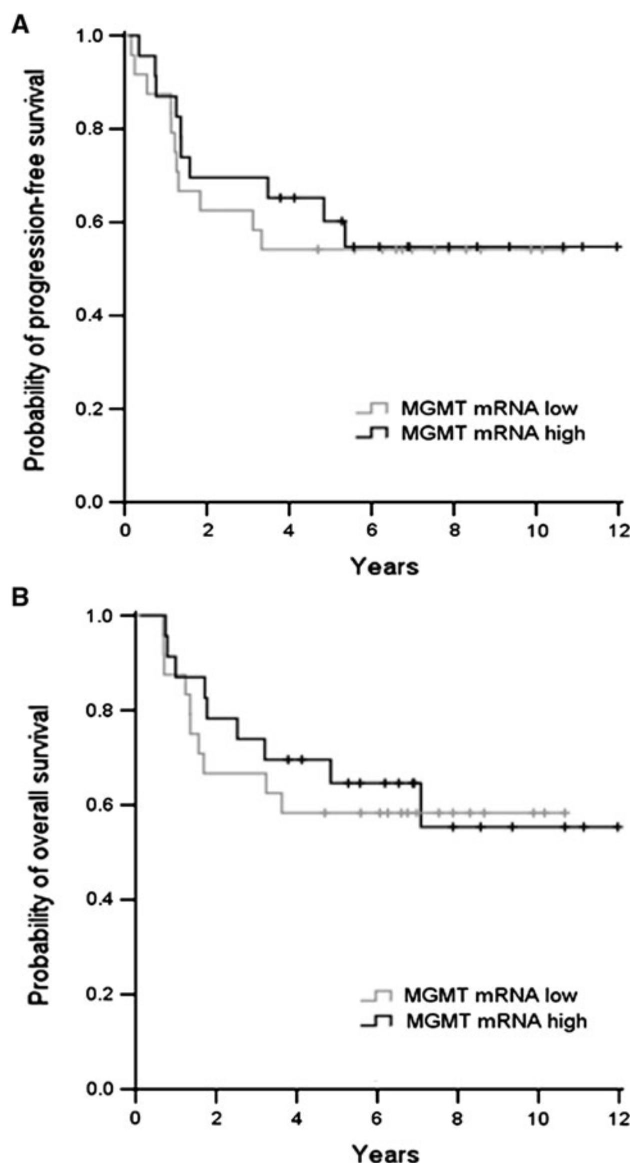

Fig. 4 PFS and OS in a cohort of uniformly treated patients with MB ($n = 47$) according to relative MGMT mRNA expression levels in primary tumor tissue

imaging; in 15 patients, primary tumor resection was incomplete. Twenty-one patients were treated within the 'maintenance arm' and 26 patients within the 'sandwich arm'. Median follow-up time of surviving patients was 7.0 years (range, 3.8–12.0). Median MGMT mRNA expression was 2.15 (range, 0.00–231.60; 25th and 75th percentiles 0.00 and 10.56, respectively). Five-year PFS of patients with MGMT expression levels lower or equal to the median was 54.2% (±10.2%), and 60.2% (±10.4%) in those with higher MGMT expression levels ($P = 0.738$) (Fig. 4a). For 5-year OS, survival probabilities were 58.3% (±10.1%) and 64.6% (±10.1%), respectively ($P = 0.720$) (Fig. 4b). In a multivariate Cox regression analysis, MGMT expression level was not shown to be an independent prognostic factor for either PFS or OS.

Discussion

Resistance to cytotoxic drugs remains a significant barrier to the successful treatment of high-risk MB patients. The contribution of MGMT to alkylating agent resistance, as well as the promise and limitations of O^6-benzylguanine in suppressing resistance, have been thoroughly examined in cell lines and xenografts derived from adult and pediatric gliomas [21, 38–41]. Also, several studies have suggested a favorable association between low MGMT levels and response to chloroethylating and methylating agents in adults and children with malignant glioma [13–16]. However, for MB, the most common malignant brain tumor in children, information about factors regulating sensitivity towards alkylating agents is largely missing.

Several methods for measuring MGMT levels within tumors have been described [41]. These include high-performance liquid chromatography (HPLC) for measuring enzymatic activity and immunohistochemistry for detecting protein level. Preusser et al. [42] recently published a large study on MGMT immunohistochemistry in 164 glioblastoma multiforme samples of glioblastoma. Using two different commercial anti-MGMT antibodies (clones MT3.1 and MT23.2), they found major observer variability and no significant association of MGMT protein expression with the MGMT promoter methylation status and patient survival. We therefore decided not to perform MGMT immunohistochemistry. Loss of MGMT expression is commonly due to *MGMT* promoter methylation [8, 13]. Epigenetic silencing of the *MGMT* gene by promoter methylation can be assessed using MSP [43]. One advantage of the MSP assay lies in the fact that detection of the methylated *MGMT* allele can be attributed solely to neoplastic cells [44]. Therefore, nontumor tissue contamination of the surgical specimen does not interfere with the result [41]. In this study, we used MSP and the more quantitative pyrosequencing technique [45] to measure *MGMT* methylation in MB. As shown in the tumor cells analyzed, the results of these two different methods correspond well. In human D425 MB cells, promoter methylation shuts down MGMT gene expression. This finding is in agreement with an earlier report showing absence of MGMT expression in D425 as determined by northern and western blot analysis [17]. In the other six human MB/PNET cell lines tested, mean percentage of methylation as determined by pyrosequencing was only 1.7–3.0%. Clearly, characteristics of cells can change when cultured for extended periods. Therefore, additional studies including primary cell cultures are needed to validate these findings.

Our in vitro findings analyzing a panel of seven human MB/PNET cell lines indicate that *MGMT* methylation and/or mRNA expression may predict response to the alkylating agents CCNU and TMZ. However, since only one MB/PNET cell line was MGMT methylated, extended studies are needed for validation. CCNU has been widely used in MB for more than two decades, whereas TMZ only started to be incorporated in first and second line protocols later [46, 47]. The IC_{50} values found for CCNU (range, 4.1–100.7 μM) and TMZ (range, 3.9–340.1 μM) correspond well with the values reported for other brain tumor cells [48–51]. Further evidence about the important role of MGMT in determining resistance in MB/PNET tumor cells was provided by our analysis of cell viability after treatment with TMZ or CCNU in combination with the specific MGMT inhibitor O^6-benzylguanine. D425, with absent MGMT expression, showed high sensitivity to TMZ and CCNU treatment. PFSK cells express low levels of MGMT, and O^6-benzylguanine-mediated sensitization to TMZ and CCNU was modest. O^6-benzylguanine strongly sensitized Med-1 and PNET-5 cells, characterized by high MGMT expression levels, to TMZ and CCNU, indicating that MGMT is a predictor of sensitivity in MB/PNET tumor cells. On the other hand, the fact that not all cells respond similarly to O^6-benzylguanine co-treatment suggest that additional factors, including MMR proteins [49], p53 [52] and apoptotic proteins, might play also a role in MB/PNET.

We then used pyrosequencing to measure *MGMT* promoter methylation in DNA isolated from 67 formalin-fixed, paraffin-embedded primary MB samples and found a wide range of *MGMT* methylation (3.7–92%, mean: 13.25%, median: 10.67%). *MGMT* methylation correlated inversely with MGMT mRNA expression. We therefore tested in a second set of primary MB, where cDNA was already available from a previous study [37], the hypothesis whether MGMT mRNA expression correlates with progression-free and/or survival outcome, but this was not the case. Clearly, this result needs to be interpreted cautiously, since the multimodal therapy of these patients included not only alkylating agents but also other cytotoxic drugs and radiotherapy.

Since *MGMT* is not (methylation values $\leq$5%) or only mildly ($\leq$10%) methylated in a subgroup of MB, methods of inhibiting or depleting MGMT need to be discussed. O^6-benzylguanine is a potent MGMT-inactivating agent that has been studied in combination with alkylating agents. It inactivates MGMT stoichiometrically [7]. However, O^6-benzylguanine lowers MGMT levels in normal cells as well, increasing toxicity from chemotherapy [41]. Interestingly, alkylating agents themselves are also MGMT-depleting agents because of their capacity to induce DNA damage [41]. MGMT depletion in the tumor could potentially be achieved with alternative dosing schedules of TMZ that deliver more prolonged exposure and higher cumulative doses than the standard 5-day regimen (150–200 mg/m^2/days for 5 days every 28-day cycle) [41, 53]. Both compressed and extended TMZ schedules have been tested preclinically and clinically. Early results suggest that continuous daily administration is more effective than a single dose [54], and more frequent administration (i.e. twice daily or every 4 h) yields the most effective depletion of MGMT activity [55, 56]. However, normal hematopoietic cells can also become sensitized. Profound lymphopenia has been reported in patients receiving extended daily treatment with TMZ at a dose of 75 mg/m^2, which can lead to opportunistic infections such as *Pneumocystis jirovecii* pneumonia [57, 58].

In summary, we found a weak, but statistically significant, correlation between *MGMT* methylation and expression in primary MB, precluding prediction of individual cases. Since differences in *MGMT* mRNA expression have not been shown to determine the clinical course in patients with MB, it is not recommended to use MGMT mRNA expression analysis to determine who should receive TMZ and/or other alkylating agents and who should not. A promising strategy to overcome MGMT-mediated chemoresistance seems to be depletion of MGMT by prolonged exposure to low doses of alkylating agents. Optimizing this approach in conjunction with modulation of dosing schedules seems a promising strategy to be tested in prospective clinical trials in order to maximize the clinical effectiveness of TMZ and other alkylating agents.

References

1. Gurney JG, Smith MA, Bunin GR (2000) CNS and miscellaneous intracranial and intraspinal neoplasms. In: SEER pediatric monograph. National Cancer Institute, pp 51–63. http://seer.cancer.gov/Publications/
2. Packer RJ (1999) Alternative treatments for childhood brain tumors. Childs Nerv Syst 15:789–794
3. Douglas JG, Barker JL, Ellenbogen RG, Geyer JR (2004) Concurrent chemotherapy and reduced-dose cranial spinal irradiation followed by conformal posterior fossa tumor bed boost for average-risk medulloblastoma: efficacy and patterns of failure. Int J Radiat Oncol Biol Phys 58(4):1161–1164
4. Gerson SL (2004) MGMT: its role in cancer aetiology and cancer therapeutics. Nat Rev Cancer 4(4):296–307
5. Tano K, Shiota S, Collier J, Foote RS, Mitra S (1990) Isolation and structural characterization of a cDNA clone encoding the human DNA repair protein for O6-alkylguanine. Proc Natl Acad Sci USA 87(2):686–690
6. Gerson SL (2002) Clinical relevance of MGMT in the treatment of cancer. J Clin Oncol 20(9):2388–2399
7. Pegg AE (1990) Mammalian O6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. Cancer Res 50(19):6119–6129
8. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG (1999) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a

common event in primary human neoplasia. Cancer Res 59(4): 793–797
9. Pegg AE, Dolan ME, Moschel RC (1995) Structure, function, and inhibition of O6-alkylguanine-DNA alkyltransferase. Prog Nucleic Acid Res Mol Biol 51:167–223
10. Gonzaga PE, Brent TP (1989) Affinity purification and characterization of human O6-alkylguanine-DNA alkyltransferase complexed with BCNU-treated, synthetic oligonucleotide. Nucleic Acids Res 17(16):6581–6590
11. Coulondre C, Miller JH (1977) Genetic studies of the lac repressor. IV. Mutagenic specificity in the lacI gene of *Escherichia coli*. J Mol Biol 117(3):577–606
12. Aquilina G, Hess P, Branch P, MacGeoch C, Casciano I, Karran P, Bignami M (1994) A mismatch recognition defect in colon carcinoma confers DNA microsatellite instability and a mutator phenotype. Proc Natl Acad Sci USA 91(19):8905–8909
13. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, Caincross JG, Janzer RC, Stupp R (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352(10):997–1003
14. Hegi ME, Diserens AC, Godard S, Dietrich PY, Regli L, Ostermann S, Otten P, Van Melle G, de Tribolet N, Stupp R (2004) Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide. Clin Cancer Res 10(6):1871–1874
15. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB, Herman JG (2000) Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 343(19):1350–1354
16. Pollack IF, Hamilton RL, Sobol RW, Burnham J, Yates AJ, Holmes EJ, Zhou T, Finlay JL (2006) O6-methylguanine-DNA methyltransferase expression strongly correlates with outcome in childhood malignant gliomas: results from the CCG-945 cohort. J Clin Oncol 24(21):3431–3437
17. He XM, Ostrowski LE, von Wronski MA, Friedman HS, Wikstrand CJ, Bigner SH, Rasheed A, Batra SK, Mitra S, Brent TP (1992) Expression of O6-methylguanine-DNA methyltransferase in six human medulloblastoma cell lines. Cancer Res 52(5):1144–1148
18. Hongeng S, Brent TP, Sanford RA, Li H, LE Kun, Heideman RL (1997) O6-Methylguanine-DNA methyltransferase protein levels in pediatric brain tumors. Clin Cancer Res 3(12 Pt 1):2459–2463
19. Rood BR, Zhang H, Cogen PH (2004) Intercellular heterogeneity of expression of the MGMT DNA repair gene in pediatric medulloblastoma. Neuro-oncology 6(3):200–207
20. Neben K, Korshunov A, Benner A, Wrobel G, Hahn M, Kokocinski F, Golanov A, Joos S, Lichter P (2004) Microarray-based screening for molecular markers in medulloblastoma revealed STK15 as independent predictor for survival. Cancer Res 64(9):3103–3111
21. Bobola MS, Silber JR, Ellenbogen RG, Geyer JR, Blank A, Goff RD (2005) O6-methylguanine-DNA methyltransferase, O6-benzylguanine, and resistance to clinical alkylators in pediatric primary brain tumor cell lines. Clin Cancer Res 11(7):2747–2755
22. von Bueren AO, Shalaby T, Rajtarova J, Stearns D, Eberhart CG, Helson L, Arcaro A, Grotzer MA (2007) Anti-proliferative activity of the quassinoid NBT-272 in childhood medulloblastoma cells. BMC Cancer 7(1):19
23. Bieche I, Nogues C, Paradis V, Olivi M, Bedossa P, Lidereau R, Vidaud M (2000) Quantitation of hTERT gene expression in sporadic breast tumors with a real-time reverse transcription-polymerase chain reaction assay. Clin Cancer Res 6(2):452–459
24. Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C (2001) An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods 25(4):386–401
25. Mengelle C, Sandres-Saune K, Pasquier C, Rostaing L, Mansuy JM, Marty M, Da Silva I, Attal M, Massip P, Izopet J (2003) Automated extraction and quantification of human cytomegalovirus DNA in whole blood by real-time PCR assay. J Clin Microbiol 41(8):3840–3845
26. Vlassenbroeck I, Califice S, Diserens AC, Migliavacca E, Straub J, Di Stefano I, Moreau F, Hamou MF, Renard I, Delorenzi M, Flamion B, DiGuiseppi J, Bierau K, Hegi ME (2008) Validation of real-time methylation-specific PCR to determine O6-methylguanine-DNA methyltransferase gene promoter methylation in glioma. J Mol Diagn 10(4):332–337
27. Mikeska T, Bock C, El-Maarri O, Hübner A, Ehrentraut D, Schramm J, Felsberg J, Kahl P, Büttner R, Pietsch T, Waha A (2007) Optimization of quantitative MGMT promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. J Mol Diagn 9(3):368–381
28. Rietschel P, Wolchok JD, Krown S, Gerst S, Jungbluth AA, Busam K, Smith K, Orlow I, Panageas K, Chapman PB (2008) Phase II study of extended-dose temozolomide in patients with melanoma. J Clin Oncol 26(14):2299–2304
29. Ringquist S, Styche A, Rudert WA, Trucco M (2007) Pyrosequencing-based strategies for improved allele typing of human leukocyte antigen loci. Methods Mol Biol 373:115–134
30. Brakensiek K, Wingen LU, Langer F, Kreipe H, Lehmann U (2007) Quantitative high-resolution CpG island mapping with pyrosequencing reveals disease-specific methylation patterns of the CDKN2B gene in myelodysplastic syndrome and myeloid leukemia. Clin Chem 53(1):17–23
31. Jones AV, Kreil S, Zoi K, Waghorn K, Curtis C, Zhang L, Score J, Seear R, Chase AJ, Grand FH, White H, Zoi C, Loukopoulos D, Terpos E, Vervessou EC, Schultheis B, Emig M, Ernst T, Lengfelder E, Hehlmann R, Hochhaus A, Oscier D, Silver RT, Reiter A, Cross NC (2005) Widespread occurrence of the JAK2 V617F mutation in chronic myeloproliferative disorders. Blood 106(6):2162–2168
32. Shaw RJ, Liloglou T, Rogers SN, Brown JS, Vaughan ED, Lowe D, Field JK, Risk JM (2006) Promoter methylation of P16, RARbeta, E-cadherin, cyclin A1 and cytoglobin in oral cancer: quantitative evaluation using pyrosequencing. Br J Cancer 94(4):561–568
33. Liu L, Schwartz S, Davis BM, Gerson SL (2002) Chemotherapy-induced O(6)-benzylguanine-resistant alkyltransferase mutations in mismatch-deficient colon cancer. Cancer Res 62(11):3070–3076
34. Grotzer MA, Eggert A, Zuzak TJ, Janss AJ, Marwaha S, Wiewrodt BR, Ikegaki N, Brodeur CM, Phillips PC (2000) Resistance to TRAIL-induced apoptosis in primitive neuroectodermal brain tumor cells correlates with a loss of caspase-8 expression. Oncogene 19(40):4604–4610
35. Cejka P, Stojic L, Mojas N, Russell AM, Heimimann K, Cannavo E, di Pietro M, Marra G, Jiricny J (2003) Methylation-induced G(2)/M arrest requires a full complement of the mismatch repair protein hMLH1. EMBO J 22(9):2245–2254
36. Kortmann RD, Kuhl J, Timmermann B, Mittler U, Urban C, Budach V, Richter E, Willich N, Flentje M, Berthold F, Slavc I, Wolff J, Meisner C, Wiestler O, Sörensen N, Warmuth-Meth M, Bamberg M (2000) Postoperative neoadjuvant chemotherapy before radiotherapy as compared to immediate radiotherapy followed by maintenance chemotherapy in the treatment of medulloblastoma in childhood: results of the German prospective randomized trial HIT '91. Int J Radiat Oncol Biol Phys 46(2): 269–279

37. Rutkowski S, von Bueren A, von Hoff K, Hartmann W, Shalaby T, Deinlein F, Warmuth-Metz M, Soerensen N, Emser A, Bode U, Mittler U, Urban C, Benesch M, Kortmann RD, Kortmann RD, Schlegel PG, Kuehl J, Pietsch T, Grotzer M (2007) Prognostic relevance of clinical and biological risk factors in childhood medulloblastoma: results of patients treated in the prospective multicenter trial HIT'91. Clin Cancer Res 13(9): 2651–2657
38. Bobola MS, Tseng SH, Blank A, Berger MS, Silber JR (1996) Role of O6-methylguanine-DNA methyltransferase in resistance of human brain tumor cell lines to the clinically relevant methylating agents temozolomide and streptozotocin. Clin Cancer Res 2(4):735–741
39. Friedman HS, Keir S, Pegg AE, Houghton PJ, Colvin OM, Moschel RC, Bigner DD, Dolan ME (2002) O6-benzylguanine-mediated enhancement of chemotherapy. Mol Cancer Ther 1(11):943–948
40. Bacolod MD, Johnson SP, Pegg AE, Dolan ME, Moschel RC, Bullock NS, Fang Q, Colvin OM, Modrich P, Bigner DD, Friedman HS (2004) Brain tumor cell lines resistant to O6-benzylguanine/1,3-bis(2-chloroethyl)-1-nitrosourea chemotherapy have O6-alkylguanine-DNA alkyltransferase mutations. Mol Cancer Ther 3(9):1127–1135
41. Hegi ME, Liu L, Herman JG, Stupp R, Wick W, Weller M, Mehta MB, Gilbert MR (2008) Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. J Clin Oncol 26(25):4189–4199
42. Preusser M, Janzer CR, Felsber J, Reifenberger G, Hamou MF, Diserens AC, Stupp R, Gorlia T, Marosi C, Heinzl H, Hainfellner JA, Hegi M (2008) Anti-O6-methylguanine-methyltransferase (MGMT) immunohistochemistry in glioblastoma multiforme: observer variability and lack of association with patient survival impede its use as clinical biomarker. Brain Pathol 18(4):520–532
43. 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(18): 9821–9826
44. Shen L, Kondo Y, Rosner GL, Xiao L, Hernandez NS, Vilaythong J, Houlihan PS, Krouse RS, Prasad AR, Einspahr JG, Buckmeier J, Alberts DS, Hamilton SR, Issa JP (2005) MGMT promoter methylation and field defect in sporadic colorectal cancer. J Natl Cancer Inst 97(18):1330–1338
45. Tost J, Gut IG (2007) DNA methylation analysis by pyrosequencing. Nat Protoc 2(9):2265–2275
46. Lashford LS, Thiesse P, Jouvet A, Jaspan T, Couanet D, Griffiths PD, Doz F, Ironside J, Robson K, Hobson R, Dugan M, Pearson AD, Vassal G, Frappaz D (2002) Temozolomide in malignant gliomas of childhood: a United Kingdom Children's Cancer Study Group and French Society for Pediatric Oncology Intergroup Study. J Clin Oncol 20(24):4684–4691
47. Baruchel S, Diezi M, Hargrave D, Stempak D, Gammon J, Moghrabi A, Coppes MJ, Fernandez CV, Bouffet E (2006) Safety and pharmacokinetics of temozolomide using a dose-escalation, metronomic schedule in recurrent paediatric brain tumours. Eur J Cancer 42(14):2335–2342
48. Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, Kondo S (2004) Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 11(4):448–457
49. Hermisson M, Klumpp A, Wick W, Wischhusen J, Nagel G, Roos W, Kaina B, Weller M (2006) O6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells. J Neurochem 96(3):766–776
50. Lewandowicz GM, Harding B, Harkness W, Hayward R, Thomas DG, Darling JL (2000) Chemosensitivity in childhood brain tumours in vitro: evidence of differential sensitivity to lomustine (CCNU) and vincristine. Eur J Cancer 36(15):1955–1964
51. Wick A, Wick W, Hirrlinger J, Gerhardt E, Dringen R, Dichigans J, Weller M, Schulz JB (2004) Chemotherapy-induced cell death in primary cerebellar granule neurons but not in astrocytes: in vitro paradigm of differential neurotoxicity. J Neurochem 91(5): 1067–1074
52. Stojic L, Brun R, Jiricny J (2004) Mismatch repair and DNA damage signalling. DNA Repair 3(8–9):1091–1101
53. Brock CS, Newlands ES, Wedge SR, Bower M, Evans H, Colquhoun I, Roddie M, Glaser M, Brampton MH, Rustin GJ (1998) Phase I trial of temozolomide using an extended continuous oral schedule. Cancer Res 58(19):4363–4367
54. Newlands ES, Blackledge GR, Slack JA, Rustin GJ, Smith DB, Stuart NS, Quarterman CP, Hoffman R, Stevens MF, Brampton MH (1992) Phase I trial of temozolomide (CCRG 81045: M&B 39831: NSC 362856). Br J Cancer 65(2):287–291
55. Newlands ES, Stevens MF, Wedge SR, Wheelhouse RT, Brock C (1997) Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials. Cancer Treat Rev 23(1):35–61
56. Spiro TP, Liu L, Majka S, Haaga J, Willson JL, Gerson SL (2001) Temozolomide: the effect of once- and twice-a-day dosing on tumor tissue levels of the DNA repair protein O(6)-alkylguanine-DNA-alkyltransferase. Clin Cancer Res 7(8):2309–2317
57. Su YB, Sohn S, Krown SE, Livingston PO, Wolchok JD, Quinn C, Williams L, Foster T, Sepkowitz KA, chapman PB (2004) Selective CD4+ lymphopenia in melanoma patients treated with temozolomide: a toxicity with therapeutic implications. J Clin Oncol 22(4):610–616
58. Ganiere V, Christen G, Bally F, Guillou L, Pica A, de Ribaupierre S, Stupp R (2006) Listeria brain abscess, Pneumocystis pneumonia and Kaposi's sarcoma after temozolomide. Nat Clin Pract Oncol 3(6):339–343