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Mismatch repair deficiency: a temozolomide resistance factor in medulloblastoma cell lines that is uncommon in *primary* medulloblastoma tumours

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BACKGROUND: Tumours are responsive to temozolomide (TMZ) if they are deficient in O^6-methylguanine-DNA methyltransferase (MGMT), and mismatch repair (MMR) proficient.

METHODS: The effect of TMZ on medulloblastoma (MB) cell killing was analysed with clonogenic survival assays. Expression of DNA repair genes and enzymes was investigated using microarrays, western blot, and immunohistochemistry. DNA sequencing and promoter methylation analysis were employed to investigate the cause of loss of the expression of MMR gene *MLH1*.

RESULTS: Temozolomide exhibited potent cytotoxic activity in D425Med (MGMT deficient, MLH1 proficient; $IC_{50} = 1.7\ \mu M$), moderate activity against D341Med (MGMT proficient, MLH1 deficient), and DAOY MB cells (MGMT proficient, MLH1 proficient). MGMT inhibitor O^6-benzylguanine sensitised DAOY, but not D341Med cells to TMZ. Of 12 MB cell lines, D341Med, D283Med, and 1580WÜ cells exhibited MMR deficiency due to *MLH1* promoter hypermethylation. DNA sequencing of these cells provided no evidence for somatic genetic alterations in *MLH1*. Expression analyses of MMR and MGMT in MB revealed that all patient specimens ($n = 74$; expression array, $n = 61$; immunostaining, $n = 13$) are most likely MMR proficient, whereas some tumours had low *MGMT* expression levels (according to expression array) or were totally MGMT deficient (3 out of 13 according to immunohistochemistry).

CONCLUSION: A subset of MB may respond to TMZ as some patient specimens are MGMT deficient, and tumours appear to be MMR proficient.

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Medulloblastoma (MB) is the most common malignant paediatric brain tumour. Standard treatment includes surgery, chemotherapy, and irradiation (for older children and adolescents). The anticancer drug that has shown promise in the treatment of recurrent paediatric brain tumours is the methylating agent temozolomide (TMZ). Already a part of standard treatment of glioblastomas (Stupp *et al*, 2005), this orally administered drug causes only mild and tolerable side-effects (Broniscer *et al*, 2007; Nicholson *et al*, 2007). In an ongoing clinical trial for recurrent paediatric brain tumours (Phase II), the efficacy of TMZ (alone) is being tested against that of intravenous carboplatin/etoposide (HIT-REZ 2005; http://clinicaltrials.gov/ct2/show/NCT00749723). Another clinical trial compares the effectiveness of the well-tested combination of TMZ and irinotecan hydrochloride with or without bevacizumab (http://clinicaltrials.gov/ct2/show/NCT01217437) in recurrent MB.

TMZ spontaneously hydrolyses to MTIC (3-methyl-(triazen-1-yl) imidazole-4 carboxamide), which then degrades into the DNA-reactive methyldiazonium cation that forms methyl adducts at *O*-6 (G), N-7 (G), N-1 (A), and N-3 (A) positions (Marchesi *et al*, 2007). The O^6-methylguanine (O^6-meG)/cytosine (O^6-meG:C) pair can result into the O^6-meG:T mismatch in the progeny DNA after a first round of replication. The mismatch repair (MMR) system, a protein complex including MSH2, MSH6, MLH1, and PMS2 proteins attempts to repair the O^6-meG:T mismatch by removing a patch of the newly synthesised strand containing thymine. However, if O^6-meG remains in the template strand, thymine could still be misincorporated opposite O^6-meG and a futile cycle of repair by the MMR system takes place during this S phase of the cell cycle. This process triggers collapsed replication fork and subsequent DNA double-strand break that then leads to G2/M arrest (Drablos *et al*, 2004). Another DNA repair protein that

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modulates TMZ activity is MGMT (O^6-methylguanine-DNA methyltransferase), which can directly excise the methyl lesion at the O^6 position of guanine (Pegg, 1990; Gerson, 2002). In theory, a defective MMR and/or presence of expression of MGMT can lead to the tumour's tolerance to TMZ. Previous studies demonstrated that increased expression (Dunn *et al*, 2009; Karayan-Tapon *et al*, 2010) or reduced promoter methylation (Dunn *et al*, 2009; Karayan-Tapon *et al*, 2010) (which also increases expression level) of *MGMT* correlated with lower overall survival of adult glioblastoma (GBM) patients whose treatment regimen included TMZ. Cahill *et al* (2007) reported that among radiotherapy/TMZ-treated GBM patients, a subset of recurrent tumours had lost the expression of the MMR protein MSH6. As clinical studies involving the use of TMZ in paediatric MB have started only fairly recently, the association between MGMT/MMR status and clinical response and outcome among TMZ-treated MB patients are yet to be reported. In this study, we aimed to investigate the impact of MGMT/MMR status on the response of MB cell lines to TMZ. We then proceeded to analyse the expression of MMR and MGMT genes and proteins by expression array (61 MB and 12 MB cell lines) and immunostaining (13 MB) to examine the potential clinical relevance of the MMR system and MGMT in MB. The underlying cause for MMR deficiency in MB cells was further explored by sequencing and methylation analysis of the *MLH1* gene.

MATERIAL AND METHODS

Cell lines and culture conditions

We used the MB cell lines DAOY (from a 4-year-old male) (Jacobsen *et al*, 1985), D341Med (from a 3.5-year-old male) (Friedman *et al*, 1988), D425Med (from a 5-year-old male) (Bigner *et al*, 1990), D556Med (from a 7-year-old female) (Aldosari *et al*, 2002), D283Med (from ascitic fluid of a 6-year-old child with metastatic MB) (Friedman *et al*, 1985), MHH-MED-1 (from the cerebrospinal fluid of a 10-year-old male) (Pietsch *et al*, 1994), MHH-MED-3 (from a 3-year-old female) (Pietsch *et al*, 1994), MHH-MED-4 (from a 4-year-old male) (Pietsch *et al*, 1994), and UW228-2 (from a 9-year-old female) (Keles *et al*, 1995). Cell lines were cultured as previously described (Hartmann *et al*, 2005; Faoro *et al*, 2011). Three cell lines (1580WÜ, 5-year-old female; MEB-MED-8S, 1-year-old male; and MEB-MED-8A, 1-year-old male) were established by one of the authors (TP) and were used as described previously (Hartmann *et al*, 2005).

Tumour samples

Two MB series were analysed: (a) 61 primary MB samples whose expression array data (Affymetrix HG U133 Plus 2.0 arrays, Santa Barbara, CA, USA) (Kool *et al*, 2008, 2012) were reanalysed for the present study, and (b) 13 archival MB tissue samples from patients diagnosed at the University Medical Center Hamburg-Eppendorf, Germany. All these archival tissue samples were reviewed by an experienced neuropathologist (CH) and the diagnosis of MB was confirmed in all. Written informed consent was obtained from all patients and/or from the parents of all patients. All cases were made anonymous before the investigation.

Drugs

Temozolomide (Schering-Plough; Kenilworth, NJ, USA) and O^6-benzylguanine (O^6-BG) (Sigma Aldrich, Basel, Switzerland) were used as described elsewhere (Faoro *et al*, 2011).

Western blot analysis

MB cells were incubated with lysis buffer (50 mM Tris HCl, pH 7.8, 0.15 M NaCl, 1% NP-40, Roche (Basel, Switzerland) complete mini EDTA-free protease-inhibitor cocktail) on ice for 30 min. Lysis was checked under the microscope and the supernatant was isolated after centrifugation. Eighty microgram of protein extracts were mixed with an equal volume of 2 × SDS loading buffer (20% glycerol, 4% SDS, 0.2 M DTT, 0.01% bromophenol blue, 0.125 M Tris HCl, pH 6.8), electrophoresed in 12% Tris-Glycine SDS polyacrylamide gel, and transferred onto Biotrace PVDF membranes (Pall Co., Ann Arbor, MI, USA). The subsequent steps for western blotting were performed using the WesternBreeze Chemiluminescent Western Blot Immunodetection kit (Invitrogen, Carlsbad, CA, USA), with the alkaline phosphatase-conjugated anti-mouse secondary antibody and CDP-Star Chemiluminescent substrate. Mouse monoclonal antibodies used were specific for MLH1 (Oncogene, Cambridge, MA, USA), MSH2 (Oncogene), MSH6 (BD Biosciences, San Jose, CA, USA), PMS2 (Oncogene), and βIII tubulin (Millipore, Billerica, MA, USA). The expression of MGMT and β-actin were analysed by western blot analysis as described previously (Faoro *et al*, 2011).

Clonogenic survival assay

To assess clonogenic survival, the number of seeded cells was adjusted to obtain around 100 colonies per cell culture dish for a given treatment (von Bueren *et al*, 2009). Where required, the specific MGMT inhibitor O^6-BG at a concentration of 25 μM was added to the cell cultures 2 h before the addition of various concentrations of TMZ. The cultures were then maintained at 37°C in a humidified atmosphere containing 5% CO_2, and allowed to grow for 9 (DAOY), 14 (D341Med), or 12 (D425Med) days, respectively. This was followed by the fixation of the cells in methanol/acetic acid (75% : 25%) and the staining with Giemsa dye. Colonies with more than 50 cells were counted. Clonogenic assays were repeated three times as independent experiments using triplicate cultures. The relative colony formation (percentage of clonogenic survival) was plotted against the drug concentrations.

MGMT and *MLH1* sequencing

Genomic DNA was extracted from DAOY, D341Med, D283Med, and 1580WÜ cells using standard procedures. The DNA fragments encompassing four exons and adjacent intronic sequences of *MGMT* (OMIM 156569) gene were PCR amplified and directly sequenced using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 3100 genetic analyser. For DAOY, D341Med, D283Med, and 1580WÜ, the genomic DNA regions covering 19 exons of *MLH1* (OMIM 120436; GenBank accession number NM_0000249) were also PCR amplified and sequenced as described recently (Buerki *et al*, 2012).

MLH1 promoter hypermethylation analysis

The promoter methylation status of *MLH1* in DAOY, D341Med, D283Med, and 1580WÜ cells was evaluated using the SALSA ME011-B1 MS-MLPA (methylation-specific, multiplex-ligation-dependent probe amplification) MMR Probemix Kit (MRC Holland, Amsterdam, The Netherlands) (Buerki *et al*, 2012). The kit included five *MLH1*-specific probes: *MLH1* 1 (237 bp; − 659 bp distance to ATG start); *MLH1* 2 (265 bp; − 383 bp distance to ATG start); *MLH1* 3 (189 bp; − 246 bp distance to ATG start); *MLH1* 4 (166 bp; − 13 bp distance to ATG start); and *MLH1* 5 (292 bp; + 208 bp distance to ATG start) (Nygren *et al*, 2005; Gylling *et al*, 2009). All reactions were carried out according to the manufacturer's protocol and fragment analysis was performed on an ABI PRISM 310 Genetic analyser (Buerki *et al*, 2012). Analyses were performed using three independent experiments.

Gene expression by microarray analysis

The gene expression data of MB tumours ($n = 61$), which are available from NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE10327) and published by Kool *et al* (2012), were reanalysed, whereas those of the 12 MB cell lines (D341Med, D425Med, D283Med, DAOY, MHH-MED-1, D556Med, 1580WÜ, MHH-MED-3, MEB-MED-8S, MHH-MED-4, MEB-MED-8A, and UW228-2) were newly generated. In both series, Affymetrix HG U133 Plus 2.0 arrays (Affymetrix) were used. Data analysis was performed using the R2 microarray analysis and visualisation platform (http://r2.amc.nl).

Immunohistochemistry

Thirteen additional MB specimens were examined immunohistochemically for MMR and MGMT protein expression using standard immunohistochemical techniques (Truninger *et al*, 2005; Menigatti *et al*, 2009). In brief, tumours were fixed in buffered formalin and embedded in paraffin. Four micrometre thick sections were mounted on glass slides coated with organosilane (DakoCytomation, Hamburg, Germany), deparaffinised, and rehydrated. Antigen retrieval was accomplished by heating the sections in a pressure cooker at 120°C for 2 min in 10 mM citrate-buffered solution (pH 6.0). DAKO (Hamburg, Germany) peroxidase blocking reagent and goat serum were sequentially used to suppress non-specific staining due to endogenous peroxidase activity and unspecific binding of antibodies, respectively. Incubations with primary monoclonal antibodies were performed as follows: anti-MSH2: 24 h at 4°C with Ab NA27 (Calbiochem, Darmstadt, Germany), 1 : 50; anti-MSH6: 24 h at 4°C with Ab 610919 (BD Biosciences), 1 : 65; anti-MLH1: 24 h at 4°C with Ab 551091 (BD Biosciences), 1 : 200; anti-PMS2: 24 h at 4°C with Ab 556415 (BD Biosciences), 1 : 85; and anti-MGMT: 24 h at 4°C with Ab MS-470-P1 (NeoMarker, Kalamazoo, MI, USA), 1 : 170. After washing, anti-mouse secondary antibodies conjugated to peroxidase labelled polymer (DAKO EnVision + kit) were applied for 30 min at RT, and the peroxidase activity was developed by incubation with 3,3′diaminobenzidine chromogen solution (DAKO). The sections were then counterstained with hematoxylin. Immunoreactivity of the tissue was verified by labelling with antibodies against the Ki-67 antigen (anti-Ki-67: 24 h at 4°C with Ab M 7240, DAKO, 1 : 125). Slides were evaluated qualitatively for nuclear staining of tumour cells.

Statistical analysis

Whenever possible, data points are reported as mean ± standard deviation (s.d.). GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA) software was used to calculate IC_{50} values and their 95% confidence intervals, and to statistically compare the fitted midpoints (log IC_{50}) of the two curves (von Bueren *et al*, 2011).

RESULTS

Temozolomide sensitivity depends on both the MMR status and on its MGMT expression

We investigated the effect of TMZ on the clonogenic survival with and without O^6-BG pretreatment in DAOY, D425Med, and D341Med cells. Results from clonogenic survival assay (CFA) indicated that D425Med cells were highly sensitive to TMZ (Figure 1). The TMZ IC_{50} values and their 95% confidence intervals for D425Med (IC_{50}: 1.705 μM), DAOY (IC_{50}: 383.8 μM), and D341Med cells (IC_{50}: 178.2 μM) are shown in Figure 1. We have previously shown that the *MGMT* gene in D425Med cells is epigenetically silenced (Faoro *et al*, 2011), explaining the cell line's sensitivity to TMZ. On the other hand, DAOY and D341Med cells

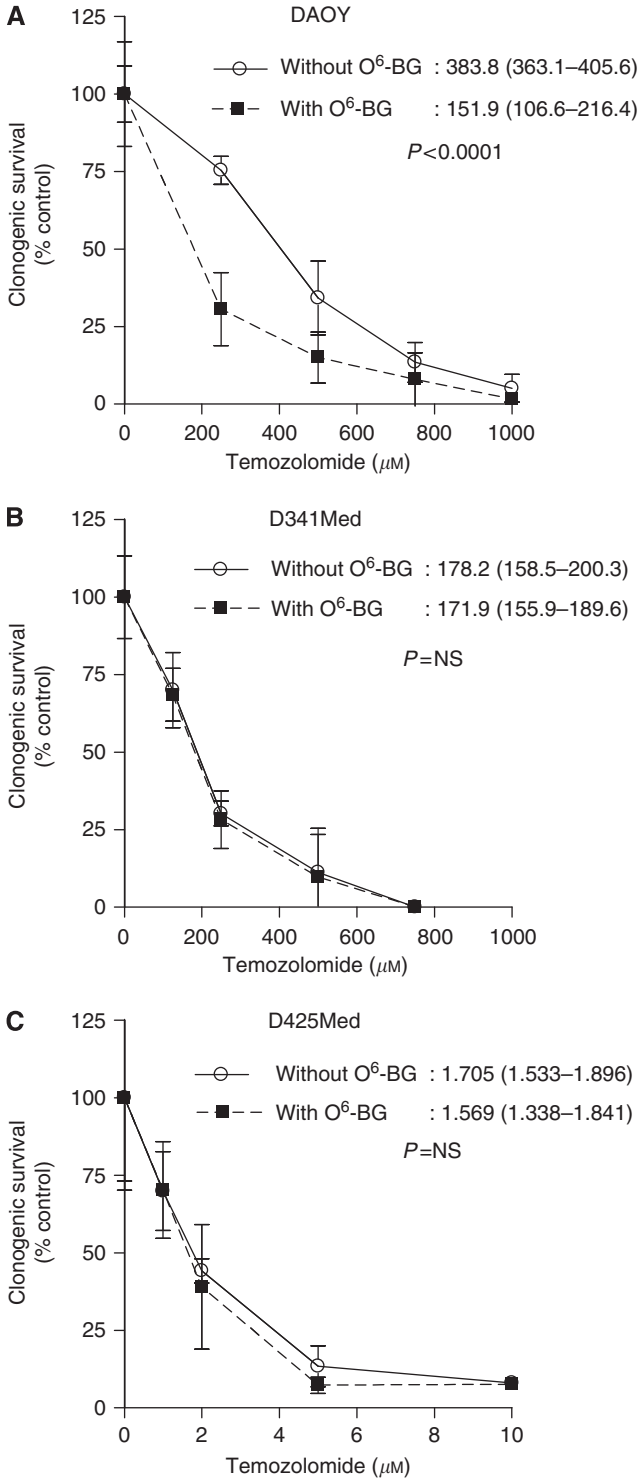

Figure 1 Effect of temozolomide alone (straight lines) or temozolomide with a 2-h pretreatment with O^6-BG (O^6-benzylguanine; dashed lines) on the clonogenic survival of the medulloblastoma cell lines DAOY (**A**), D341Med (**B**), and D425Med (**C**). Single cells were seeded into culture dishes and treated with the denoted concentrations of temozolomide in absence or presence of O^6-BG and then allowed to further grow for 9 (DAOY), 14 (D341Med), or 12 (D425Med) days, respectively. Data are presented as the mean ± standard deviation (s.d.) of three independent experiments performed in triplicates. The IC_{50} values and their 95% confidence intervals were calculated from the regression curve and are indicated for each data set for temozolomide alone and temozolomide with a 2-h pretreatment with O^6-BG for DAOY, D341Med, and D425Med cells. Differences between the two curves are represented by *P*-values ($P < 0.001$, $P < 0.01$, $P < 0.05$; NS, not significant).

expressed MGMT (Faoro *et al*, 2011), consistent with their tolerance to TMZ. Upon addition of the MGMT inhibitor O^6-BG, the depletion of MGMT protein expression was evident for both DAOY and D341Med cells (data not shown). However, as indicated in Figure 1, O^6-BG addition was able to potentiate the cytotoxic activity of TMZ in DAOY (TMZ IC_{50} value of 151.9 μM, significantly lower than the basal IC_{50} of 383.8 μM; $P<0.001$), the most TMZ-resistant MB cell line analysed in our study. In contrast, no significant increased cytotoxic activity of TMZ was evident in D425Med and D341Med cells upon O^6-BG addition. This observation was expected for the MGMT-deficient D425Med cells. For the MGMT-proficient D341Med cells, these results were quite surprising. We first considered the possibility that the D341Med cells used in these experiments carried a mutated yet immuno-detectable MGMT protein, that is, a mutant protein resistant to inhibition by O^6-BG, such as those variants previously reported in BCNU/O^6-BG-resistant MB sublines (Bacolod *et al*, 2002, 2004). Sequencing did not reveal any evidence for somatic genetic alterations in *MGMT*, when assessing D341Med, DAOY and D425Med cells. However, when we analysed the MMR status by western blot (Figure 2), losses of MLH1 and PMS2 expression were found in D341Med cells, while all the MMR proteins investigated were normally expressed in DAOY.

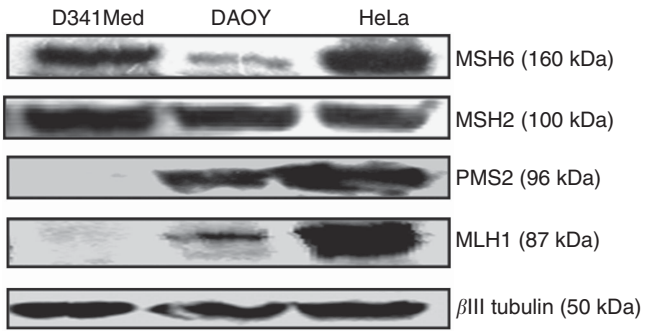

Figure 2 Analysis of protein expression of mismatch repair polypeptides MSH6, MSH2, PMS2, and MLH1 in D341Med, and DAOY cells by western blotting (βIII Tubulin expression used as loading control). Positive controls were protein extracts from HeLa cells. Representative blot (two independent experiments) is shown.

MB tumours are MMR proficient, some MB cells are MMR deficient, and the majority of MB tumours and MB cells are MGMT proficient

The loss of MMR function in one of three MB cell lines, prompted us to investigate the frequency of MMR deficiency in MB tumours. We analysed the expression levels of the four MMR genes among 61 primary MB tumours. The clinical, histological, and molecular subgroup (WNT, SHH, group 3 (GRP3) and group 4 (GRP4)) characteristics of the 61 patients are described elsewhere (Kool *et al*, 2012). Included as reference were the expression data of nine normal cerebellum (CB; without information about age) samples which were assessed also by Affymetrix HG U133 Plus 2.0 array by Roth *et al* (2006), and data were available from NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; accession number GSE3526). As shown in Figure 3, the expression levels of *MLH1*, *MSH2*, and *MSH6* across 61 tumour samples (and all four molecular subtypes) were higher compared with the MMR gene expression assessed in the CB samples. The expression of *PMS2* in primary MB tissues was within the range of the reference CB. Taken together, these data suggest that MMR defects associated with loss of expression of MMR genes are absent or rare in MB tumours. We then examined the expression level of MMR genes among 12 established MB cell lines; including the three cell lines used in the CFAs. Expression array analysis (Figure 4) indicated that of the 12 cell lines, 3 had undetectable expression of the MMR

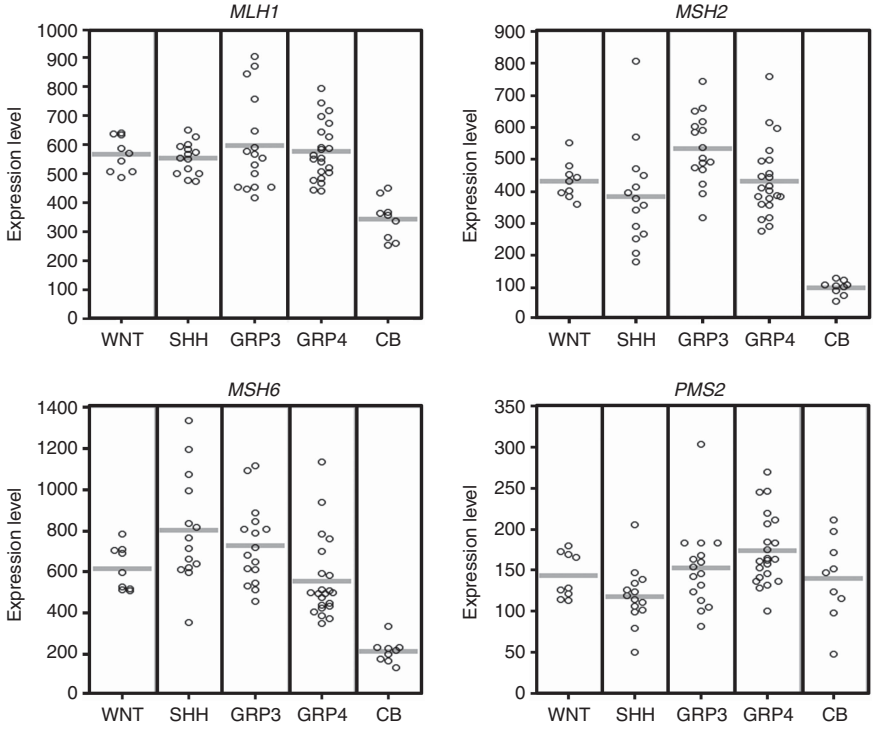

Figure 3 The expression levels (Affymetrix HG U133 Plus 2.0 arrays) of the mismatch repair genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* in 61 medulloblastoma (Kool *et al*, 2012) grouped into the four molecular subtypes (Taylor *et al*, 2012) of medulloblastoma (WNT, SHH, Group 3 (GRP3) and Group 4 (GRP4)). Also included are the gene expression levels of mismatch repair genes for nine normal cerebellum (CB) samples published previously (Roth *et al*, 2006).

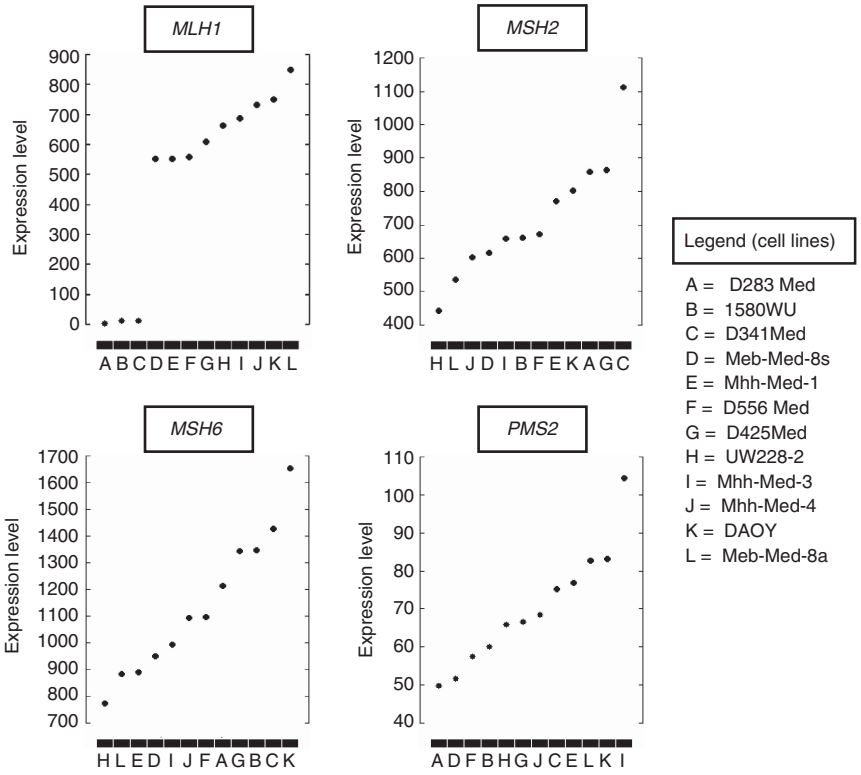

Figure 4 The expression levels (Affymetrix HG U133 Plus 2.0 arrays) of mismatch repair genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* in 12 medulloblastoma cell lines.

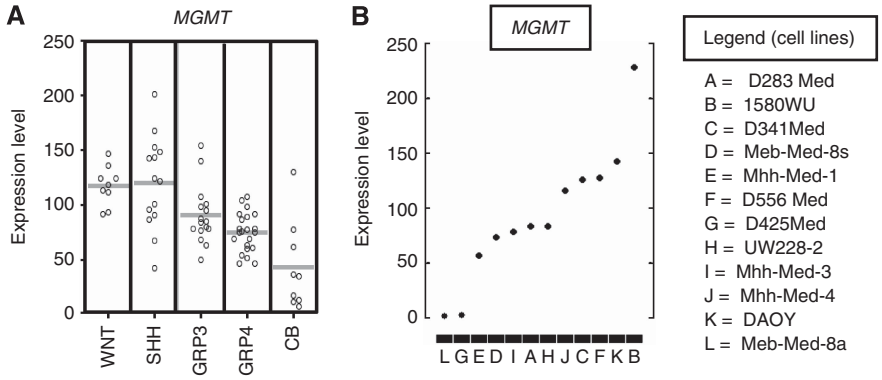

Figure 5 (**A**) The expression levels (Affymetrix HG U133 Plus 2.0 arrays) of *MGMT* in 61 medulloblastoma (Kool *et al*, 2012) grouped into the four molecular subtypes (Taylor *et al*, 2012) of medulloblastoma (WNT, SHH, Group 3 (GRP3), and Group 4 (GRP4)) compared with nine samples of normal cerebellum (CB), (**B**) *MGMT* expression levels of 12 medulloblastoma cell lines.

gene *MLH1*: D341Med, D283Med, and 1580WÜ. This confirmed what we found in D341Med cells by western blot: loss of the MLH1 protein expression (Figure 2). In D341Med cells, the non-detection of the PMS2 protein (even though the mRNA is expressed) can be explained by its degradation in the absence of the heterodimeric partner MLH1 (Figure 4) (Yao *et al*, 1999). Expression array analysis also showed that *MGMT* expression is detectable in all of the 61 MB tumours with transcription levels equal to or higher compared to those of CB samples (Figure 5A). Microarray analysis of the 12 MB cells revealed that only 2 cell lines (D425Med and Meb-Med-8a) did not express *MGMT* mRNA (Figure 5B). Unfortunately, tissue blocks of the 61 MB tumours used in the microarray study were not available for the analysis of the MMR protein expression. Therefore, we tested an additional 13 MB tumours (Table 1) by immunohistochemistry and found that all of them were positive for the presence of MSH2, MSH6, MLH1, and PMS2. A representative example is shown in Figure 6. Apparently, three of the 13 tumours (23%) showed complete loss of MGMT protein expression (Table 1).

Mechanism of *MLH1* silencing in D341Med, D283Med, and 1580WÜ cells

We then asked whether the lack of expression of *MLH1* in D341Med, D283Med, and 1580WÜ cell lines can be explained by hypermethylation of the gene's promoter region. Using SALSA ME011-B1 MMR assay, we were able to demonstrate that all three cell lines exhibited extensive CpG island methylation in at least four of five *MLH1* promoter regions that were investigated (Table 2). This methylation pattern appeared to be sufficient for

Table 1 Disease characteristics of 13 medulloblastoma patients analysed for expression of mismatch repair polypeptides MLH1, MSH2, MSH6, and PMS2 by immunohistochemistry

Characteristics	
Sex	
Male	8 (61.5%)
Female	5 (38.5%)
Age (years)	
Median age at diagnosis (range) of 12 patients	10.7 (2.9–64.8)
NA ($n=1$)	
Histology	
Classic MB	11 (84.6%)
Desmoplastic MB	2 (15.4%)
Large cell/anaplastic MB	0 (0%)
NA	0 (0%)
Extent of metastasis	
M0	8 (61.5%)
M1	0 (0%)
⩾M2	4 (30.8%)
NA	1 (7.7%)

Abbreviations: MB = medulloblastoma; M1 = presence of tumour cells in the cerebrospinal fluid at time of diagnosis; ⩾M2 = metastatic disease (macroscopic) at initial presentation on MRI of the brain (M2), spine (M3), or extraneuronal metastases (M4); NA = not available.

silencing of the *MLH1* expression (Figure 4). In contrast, DAOY (which is positive for MLH1 expression) was methylated in only one CpG site (Table 2). DNA sequencing also indicated that none of the aforementioned cell lines (including DAOY) were mutated in *MLH1*.

DISCUSSION

The two important molecular predictors of TMZ efficacy are MGMT expression and MMR status. Using MB cell line models, we were able to demonstrate that TMZ is most effective when the MB cells have a functioning MMR, in addition to low level of MGMT expression (whose correlation to TMZ sensitivity in MB has been previously demonstrated through *in vitro* viability assays (Daniel *et al*, 2010; Faoro *et al*, 2011) and xenograft studies (Daniel *et al*, 2010)). For cells with defective MMR, MGMT depletion (e.g., by O^6-BG) will not potentiate TMZ activity, as we have shown in this study, and has been reported previously using colon cancer cell lines (Liu *et al*, 1996).

Thus far, there is limited information available regarding the prevalence and clinical relevance of MMR deficiency among MB. A recent report by Viana-Pereira *et al* (2009) indicated that 4 out of 36 (11%) MB tumours had microsatellite instability (the resulting phenotype of defective MMR). However, an earlier report found that *MLH1*, *MSH2*, and *PMS2* (*MSH6* was not probed) were expressed in all of 22 MB analysed by western blotting (Lee *et al*,

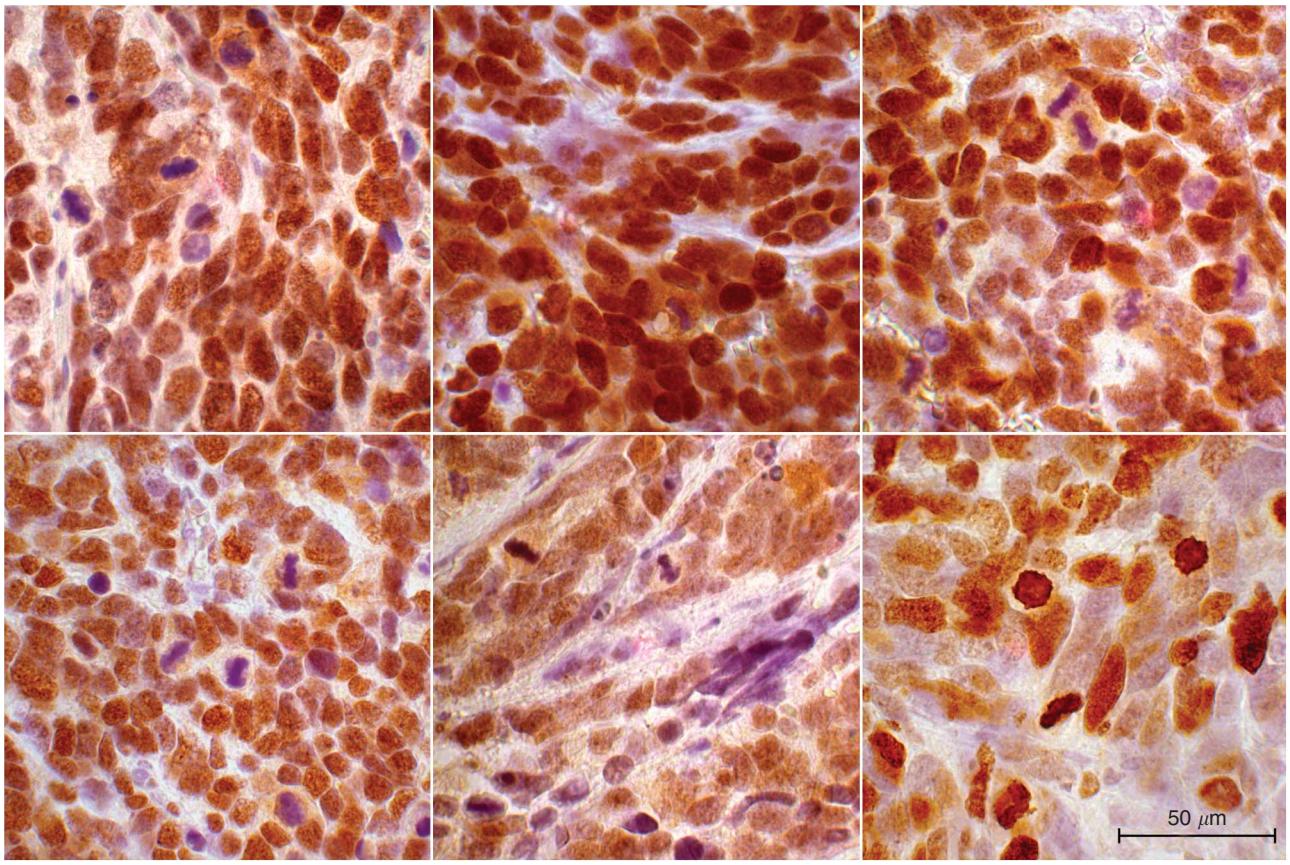

Figure 6 Immunohistochemistry of MMR proteins, MGMT, and Ki-67 antigen in 13 medulloblastoma tissues samples. Demonstration of nuclear expression of MLH1 (upper left), MSH2 (middle), MSH6 (upper right), PMS2 (lower left), MGMT (middle), and Ki-67 antigen (lower right) in a medulloblastoma from a 10-year-old girl (chromogen diaminobenzidine, scale bar applies to all pictures).

Table 2 *MLH1* promoter methylation of DAOY (included as control), and MB cell lines (D283Med, 1580WÜ, and D341Med) with absence of *MLH1* expression as determined by gene expression profiling

	MLH1 promoter methylation status (% of methylated probe)						
Cell line	**MLH1 probe 07187-L07710 A-region**	**MLH1 probe 06221-L01747 B-region**	**MLH1 probe 06222-L07712 C-region**	**MLH1 probe 01686-L15580 D-region**	**MLH1 probe 02258-L01745 Intron 1**	**Number of methylated probes**	**Inter-pretation**
DAOY	69	0	10	0	0	1 Out of 5	Not methylated
D283Med	97	100	84	95	97	5 Out of 5	Methylated
1580WÜ	100	100	100	0	55	4 Out of 5	Methylated
D341Med	100	100	100	100	84	5 Out of 5	Methylated

Abbreviation: MB = medulloblastoma.

1998). In the present study, we demonstrated that all of the 74 MB tumours (61 analysed by expression profiling and 13 by immunostaining) seem to be MMR proficient. In addition, MMR deficiency has been described in only a small fraction of malignant glioma in the paediatric age group (Vladimirova *et al*, 2008; Pollack *et al*, 2010). Furthermore, very rare cases of MMR deficiency originating from biallelic germline mutations of a MMR gene have been reported (Poley *et al*, 2007; Scott *et al*, 2007). Taken collectively, these studies point to the low prevalence of MMR deficiency in MB tumours. Indeed, the transcript levels of MMR genes *MLH1*, *MSH2*, and *MSH6* were clearly higher in MB tumours when compared with CB samples. The transcript levels of *PMS2* in MB tumours (expression levels were slightly higher when compared with CB tissues) need to be interpreted with caution as the probe seems to detect *PMS2* and *PMS2CL* (the probe sets 209805_at and 221206_at can detect both *PMS2* and *PMS2CL* mRNAs; noted in affymetrix.com).

This current study also showed that, in contrast to the *primary* MB tumours, 3 out of 12 MB cell lines had defective MMR system due to promoter hypermethylation of the *MLH1* gene. As we have not been able to obtain primary tumour specimens from which the cell lines were established, we can not conclude whether this epigenetic event occurred in the primary tumours or during the establishment and propagation of the corresponding cell lines. There are possible explanations for the observation that 3 out of 12 MB cell lines are, due to MLH1 promoter hypermethylation, MMR deficient, while *primary* MB tumours are MMR proficient. First, the cancer cell lines may have been selected from a sub-population of cancer cells specifically adapted to growth in tissue culture. Some cells from the primary tumour may have possessed *MLH1* promoter hypermethylation, thus MMR-deficiency. Although the great majority of the tumour cells were MMR proficient, those MMR-deficient cells might have been selected during the establishment of the tumour cell line. Another possible explanation is that at least part of the methylations might have been acquired *in vitro* during cell propagation.

In line with our observation regarding MB tumours and cell lines is the fact that colorectal cancer cell lines display higher rates of methylations (Suter *et al*, 2003) when compared with *primary* colorectal cancers (Lengauer *et al*, 1998) (e.g., *MLH1* promoter methylation was detected in 30% of colorectal cancer cell lines (Suter *et al*, 2003)). Thus, it seems that MB tumours are, like the great majority of cancers rarely MMR deficient (Lengauer *et al*, 1998). Some MB cell lines are MMR deficient. So how does MMR deficiency confer an advantage on cells *in vitro*? In MMR-deficient cells, mutations in oncogenes and tumour suppressor accumulate more easily, thus resulting in clonal expansion. These cells might be more conducive to transformation into culture. This inconsistency between tumours and cell lines highlight the limitations when using cancer cell lines as a model to study MB or other cancers. Differences between primary tumours and cancer cell lines have been reported for gene copy numbers, as several cancer-related genes show higher amplification and deletion frequencies in cell lines than tumours of their respective histology as demonstrated in MB for several genes including *c-MYC* gene amplification (Grotzer *et al*, 2001).

The expression of MGMT (detected at the mRNA level, the protein level, activity, or indirectly as promoter methylation) is detectable in many MB (Hongeng *et al*, 1997; Bobola *et al*, 2001; Rood *et al*, 2004; Faoro *et al*, 2011). An important issue is whether data from microarray analysis (which detects mRNA expression) and those results from immunohistochemical analysis (which detects protein expression) can be interpreted similarly. According to our gene expression analysis, *MGMT* expression seems to be present in primary MB, and expression values of $\sim$20% of the tumours are rather low (similar *MGMT* mRNA expression levels as CB samples). We would in general expect higher *MGMT* transcript levels – similar transcript levels as observed for MMR genes *MLH1*, *MSH2*, and *MSH6* – in MGMT-proficient tumours when compared with CB samples. Silber *et al* (1993) showed a generally higher MGMT expression in brain tumours compared to adjacent normal brain tissues. Whether in those tumours with low *MGMT* mRNA expression the protein might be detectable in tumour cells by immunohistochemistry is questionable. Moreover, we need to consider that we can not exclude the possibility that the low *MGMT* expression assessed at the transcript level by microarray analysis could be the result from the contamination with tumour stroma cells or non-tumour cells expressing *MGMT* mRNA. Of note, a snap-frozen MB tumour sample was included in the microarray study when $\geqslant$70% of the sample contained tumour cells (Kool *et al*, 2008, 2012). The fact that 2 out of 12 MB cell lines were MGMT deficient is providing further evidence that a small proportion of MB tumours might be MGMT deficient. In addition, there is marked intratumoral heterogenicity for MGMT immunoreactivity in the brain tumours including MB (Rood *et al*, 2004). Ideally, we would have analysed the two different cohorts by both techniques (microarray analysis and immunohistochemistry) and this is a limitation of our study. As we do not have the tissue blocks of the primary MB used in the microarray study, and as we have very limited amounts of tissues from the tumours analysed by immunohistochemistry, we were unable to assess MGMT/MMR proteins and mRNA expression in the two patient cohorts. Although one would expect concordance when comparing *MGMT* promoter methylation status, *MGMT* mRNA expression, and MGMT protein expression, discrepancies have been reported in several tumour entities including MB (Rood *et al*, 2004) and these results highlight the limitations of the methods used to date to assess MGMT protein/mRNA expression and promoter methylation. Given the low frequency of MMR deficiency in MB tumours (likely no one in our series ($n = 74$)), MGMT expression level seems to be a more relevant factor mediating tolerance to TMZ in MB and could therefore have a role as predictive/prognostic factor. Studies of GBM patients suggest that the level of MGMT expression is a predictor of TMZ response and outcome (i.e., higher MGMT expression correlated to poorer overall survival rate) (Dunn *et al*, 2009; Karayan-Tapon *et al*, 2010). However, recent reports

investigating a limited number of paediatric brain tumour patients (including MB) (Sardi *et al*, 2009; Faoro *et al*, 2011) point to the weakness of such an association (Sardi *et al*, 2009; Faoro *et al*, 2011). Several explanations are possible. First, multimodal therapy of MB patients includes not only alkylating agents but also other cytotoxic drugs and radiotherapy. Second, most of the reported MGMT expression levels on MB were performed in *primary* tumours before any chemotherapy. It is possible that MGMT expression is further upregulated during the course of treatment. This has been demonstrated *in vitro*, where continuous exposure of O^6-BG alkylating drugs such as BCNU led to further upregulation of MGMT (and BCNU resistance) in MB cell lines (Bacolod *et al*, 2002, 2004). Zhang *et al* (2010) applied a similar approach to a glioma cell line (U373VR) (continuous exposure to TMZ) and also detected MGMT upregulation in the resulting TMZ-resistant line. Third, just like MGMT upregulation, MMR deficiency may arise during the course of TMZ treatment. In the aforementioned study by Zhang *et al* (2010), a glioma sub-line which acquired TMZ resistance also showed decreased expression of MSH6. Moreover, recent studies indicate that MMR gene abnormalities can be acquired in malignant glioma with initially intact MMR as a somatic mutation (*MSH6*) after treatment with alkylating agents (Pollack *et al*, 2010). Finally, another resistance mechanism may be involved. For instance, a third DNA repair mechanism, base excision repair (BER), has been shown to contribute to TMZ resistance in cancer cells (Marchesi *et al*, 2007). The rational for this finding is that TMZ also causes other DNA damages, such as N^7-meG and the highly cytotoxic N^3-meA, which are repaired by BER.

The depletion of MGMT by adding O^6-BG before TMZ therapy proved to be effective in potentiating the anti-cancer toxicity against MB in preclinical studies (Friedman *et al*, 1995; Bobola *et al*, 2005). In a clinical trial investigating a combination of TMZ/O^6-BG in paediatric patients with recurrent brain tumours (including MB) (Broniscer *et al*, 2007) showed modest anti-tumour activity of the drug combination (Broniscer *et al*, 2007). The use of O^6-BG, however, bears the risk of serious complications since O^6-BG can also potentiate TMZ in killing normal bone marrow cells. Owing to hematopoietic toxicity and lack of efficacy, the combination of O^6-BG/BCNU was not further tested in clinical trials of paediatric CNS tumours (Adams *et al*, 2008). Moreover, O^6-BG can elevate the mutagenicity of TMZ, as it inhibits the very protein (MGMT) that repairs its mutagenic adduct (O^6-meG) (Cai *et al*, 2000). Accordingly, continuous exposure of MB cells to O^6-BG and BCNU may result in the development of BCNU-resistant cells that have mutated MGMT. The mutant MGMT, it turned out, was inhibited by O^6-BG much less effectively (Bacolod *et al*, 2002, 2004).

In summary, certain molecular factors such as MGMT and MMR expressions/activities may influence the efficacy of TMZ in MB. Our data demonstrate that a subset of MB may successfully respond to TMZ as some MB tumours are MGMT deficient, and MB tumours fortunately appear to be MMR proficient. However, as MMR deficiency is evident in 3 out of 12 MB cell lines (in all cases due to transcriptional silencing of *MLH1* gene), we may speculate that this defect can arise during/after treatment of MB patients, a hypothesis that needs to be tested by investigating recurrent MB for these DNA repair proteins. Thus, it would be preferable to evaluate MGMT and MMR in tumour tissues obtained from surgery of the relapse before treatment with TMZ.

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