Temozolomide-induced severe myelosuppression: analysis of clinically associated polymorphisms in two patients

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Genotyping of putative determinants of temozolomide (TMZ)-induced life-threatening bone marrow suppression was performed in two patients with glioma treated with adjuvant TMZ and radiation therapy. DNA was extracted from the patients' mononuclear cells and genotyping of O^6-methylguanine-DNA-methyltransferase (*MGMT*), multidrug resistance (*MDR1*; also known as *ABCB1*), *NQO1*, and *GSTP1* genes and analysis for the epigenetic silencing of specific *MGMT* gene promoters were carried out to evaluate the possible genetic determinants of increased risk of severe TMZ-induced myelosuppression. Although both patients were heterozygous for all ABCB1 single nucleotide polymorphisms and for rs12917 and rs1803965 in the *MGMT* gene, patient 1 was heterozygous for rs1695 in *GSTP1* and rs2308327 in the *MGMT* gene. This patient also exhibited GG genotype for the MGMT single nucleotide polymorphisms, rs2308321, which is noteworthy for its 0.7% frequency globally. Epigenetic silencing of *MGMT* gene was not detected in either patient. Two single nucleotide polymorphisms identified in patient 1 (missense I143V and K178R polymorphisms; rs2308321 and rs2308327, respectively) have recently been shown to correlate with an increased risk of severe TMZ-induced myelosuppression. The polymorphisms identified in patient 2 have not been associated with an increased risk of severe TMZ-induced myelosuppression. Genotyping analyses of larger patient populations administered TMZ are required to validate the genetic determinants of severe TMZ-induced myelosuppression. *Anti-Cancer Drugs* 22:104–110 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The current standard of care for patients with newly diagnosed glioblastoma (GBM) was established by data from a landmark trial published in 2005. The addition of temozolomide (TMZ) 75 mg/m^2/day for 42 days to adjuvant radiotherapy administered to newly diagnosed patients with GBM showed statistically significant improvement in median and 2-year survival rates compared with radiotherapy alone [1]. A recent update analyzing 5-year survival rates indicates that the survival benefit is maintained [2]. During the initial 7-week concomitant chemoradiotherapy, patient tolerability was reported to be acceptable (incidence of grade-3 or grade-4 neutropenia and thrombocytopenia were 4 and 3%, respectively) [1].

In view of that low rate of hematological toxicity, we thought it unusual when within 3 months two patients receiving concurrent low-dose TMZ and radiotherapy were hospitalized for empiric treatment of febrile neutropenia and severe bone marrow suppression. Both patients developed prolonged grade-4 neutropenia (duration between 17 and 21 days) and thrombocytopenia (duration between 29 and 43 days).

Our initial literature review yielded two case reports of patients with GBM who developed prolonged, severe bone marrow suppression during the administration of concurrent radiotherapy and low-dose TMZ, one case of aplastic anemia diagnosed after completion of the low-dose TMZ radiotherapy regimen and one case of aplastic anemia diagnosed after completion of the fourth cycle of adjuvant 5-day high-dose TMZ [3–5]. Singhal *et al.* [3] proposed that genetic polymorphisms of the DNA mismatch repair enzymes may be a risk factor for TMZ-induced myelosuppression.

Several studies have suggested that development of severe myelosuppression might be caused by decreased expression or inactivation of the DNA repair protein, O^6-methylguanine-DNA-methyltransferase (MGMT) in peripheral blood mononuclear cells [6–8]. The *MGMT* gene has been shown to contain several single nucleotide polymorphisms (SNPs) that are associated with altered activity *in vivo* [9,10]. In addition, epigenetic silencing of

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specific gene promoters has been associated with decreased MGMT expression and increased cellular TMZ-induced cytotoxicity [11–14]. In fact, such epigenetic silencing of the *MGMT* gene in the tumor is crucial in differentiating the patients most likely to benefit from TMZ [13,15,16]. Chakravarti *et al.* [17] showed significant enhancement of radiation–TMZ cytotoxicity in MGMT-deficient compared with MGMT-proficient GBM cell lines.

Two recently published reports have described gene polymorphisms affecting the risk of TMZ-induced myelosuppression. Nagane *et al.* [18] reported a case of TMZ-induced myelosuppression and analyzed the MGMT status in the patient's GBM, peripheral blood leukocytes, and bone marrow cells. Although the promoter region of *MGMT* gene in the tumor was methylated, peripheral blood and bone marrow cells were unmethylated, suggesting differential sensitivity between tumor and myeloid cells.

Armstrong *et al.* [19] reported that in addition to MGMT polymorphisms, polymorphisms in genes *NQO1* and *GSTP1* were found to correlate with severe myelotoxicity in glioma patients. The *NQO1* gene is a member of the NAD(P)H dehydrogenase (quinone) family and encodes a cytoplasmic 2-electron reductase. Glutathione S-transferases are a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. Therefore, both NQO1 and glutathione S-transferases pi 1 (GSTP1) in their normal state should be protective against myelosuppression.

Multidrug resistance genes (*MDR1*; also known as *ABCB1* genes), which encode for P-glycoprotein drug efflux pumps, are also known to influence cell susceptibility to chemotherapy-induced cytotoxicity [20]. Recently, Schaich *et al.* [21] reported that polymorphisms in the *ABCB1* gene that encodes for the MDR1 efflux pump might be related to the efficacy of TMZ therapy in GBM patients. Although data on ATB-binding cassette subfamily B member 1 (ABCB1) polymorphisms and risk for vincristine-induced and docetaxel-induced hematopoietic toxicities exist, no data describing the effects of MDR1 polymorphisms on TMZ-induced hematopoietic toxicity have been published [22,23].

We report the results of MGMT, ABCB1, NQO1 and GSTP1 genotyping and analysis for epigenetic silencing of specific *MGMT* gene promoters for two patients who developed life-threatening TMZ-induced myelosuppression.

Case history 1

A 40-year-old Caucasian male patient with recurrent oligodendroglioma, undergoing combination TMZ radiotherapy, presented to the emergency room with sporadic fevers associated with chills and sweats for several days. He also reported easy bruisability and pain localized to an area of cellulitis on the left lateral thigh. A complete blood count showed hemoglobin (7.4 g/dl), white blood cell count (WBC) of 500/μl, absolute neutrophil count (ANC) of less than 100/μl, and platelet count of 7000/μl. Physical examination showed stomatitis and 1 to 2 + pretibial edema of the left leg, painful cellulitis, and possible necrotizing fasciitis of the left lateral and anterior thigh.

Outpatient medications included dexamethasone (3 mg twice daily), famotidine (20 mg twice daily), and levetiracetam (500 mg twice daily). In addition, he had been receiving concurrent radiation and TMZ therapy (2 Gy per day and 75 mg/m^2/day). The patient's WBC was within normal limits on initiation of treatment. The TMZ was discontinued on day 23, after his weekly WBC dropped from 7.6 to 4.9/μl. A week later the WBC was 0.8/μl and platelet count was 14 000/μl. The patient refused a platelet transfusion; he presented to the emergency room the following day as detailed above and was admitted to the hospital.

On admission, he received red blood cell and platelet transfusions and was administered empiric intravenous antimicrobials and subcutaneous sargramostim (500 mcg). The patient was taken to surgery the next day for incision, drainage, and debridement of the lesion on the left thigh. The pathology report ruled out necrotizing fasciitis. The patient's temperature normalized on day 5 after beginning antibiotic therapy.

He remained neutropenic with an ANC less than 100/μl for 18 days and an ANC less than 500/μl for 21 days. He was discharged 20 days after admission with a WBC of 1700/μl and ANC less than 500/μl with instructions to take levofloxacin (750 mg) orally daily for 3 days and to continue sargramostim (pending recovery of his WBC). The sargramostim was discontinued 6 days later after his ANC was more than 2000/μl. After sargramostim was discontinued his ANC decreased to less than 2000/μl and remained below that level for 4 months.

The patient's medical history was significant for an earlier episode of TMZ-induced neutropenia. That episode occurred 16 months earlier after completion of the first 5-day cycle of conventional dose of TMZ administered as adjuvant therapy after the initial diagnosis of oligodendroglioma. At that time, he was admitted to the hospital for treatment with empiric antibiotics and filgrastim. His neutropenia persisted for 27 days. However, on the completion of the filgrastim his WBC dropped to 3200/μl and did not normalize until 3 months later. No further TMZ was administered until his recurrence when he began his radiation and TMZ administration after undergoing gross total resection of the recurrent disease.

Case history 2

A 55-year-old Caucasian female patient with GBM, undergoing concurrent TMZ radiotherapy, presented to the emergency room with a 12-hour intermittent history

of fever with shaking chills. A complete blood count showed hemoglobin 5.6 g/dl, WBC of 500/μl, ANC less than 100/μl, and platelet count of 30 000/μl. Physical examination was unremarkable except for several areas of stomatitis on her tongue and significant alopecia. She was admitted to the hospital and treated with empiric antibiotic therapy and filgrastim (480 mcg daily). Platelet and red blood cell transfusions were also administered. Her temperature exceeded 101°F for the subsequent 5 days; blood cultures drawn on each day showed no growth. Acyclovir was initiated after cultures of the tongue lesions were positive for herpes simplex type I.

Outpatient oral medications included, dexamethasone (2 mg twice daily), famotidine (20 mg twice daily), levetiracetam (500 mg twice daily), vitamin D (1000 units daily), losartan (100 mg daily), fluconazole (100 mg daily), aspirin (81 mg daily), nifedipine (30 mg daily), pilocarpine (5 mg three times daily) for dry mouth, docusate sodium (100 mg twice daily), multivitamin (once daily), and hydrocodone/acetaminophen (5/500 mg every 3 h as needed).

The patient's medical history was significant for a subtotal resection of a right frontal mass, with pathology showing a WHO grade-4 GBM. She subsequently began concurrent radiation and TMZ therapy (2 Gy per day and 75 mg/m^2/day). Her WBC was within normal limits on initiation of treatment and was repeated weekly. On day 36 of the administration of TMZ the patient's CBC showed a WBC of 0.4/μl and platelet count of 45 000/μl. TMZ was discontinued the following day when a repeated CBC confirmed grade-4 myelosuppression. She presented to the emergency room and was admitted to the hospital as described above.

The patient's temperature normalized on the day 6 after beginning antibiotics. However, she remained neutropenic with an ANC less than 100/μl for 15 days and an ANC less than 500/μl for 17 days. She was discharged on March 19, 2009 with a WBC of 2100/μl and ANC of 630/μl with instructions to continue filgrastim for an additional 2 days. Her WBC and differential remained within normal limits after filgrastim was discontinued.

Several weeks after discharge from the hospital whole blood specimens were collected from both patients, and mononuclear cells were isolated for genotyping analysis of MGMT, ABCB1, NQO1, and GSTP1 families performed to probe for markers for increased risk of TMZ-induced bone marrow suppression.

Methods

This retrospective pilot study was carried out in the outpatient clinic of a community oncology clinic and cancer research institution (Roger Maris Cancer Center, MeritCare Health System, Fargo, ND; Analytical Instrumentation Laboratory for Pharmacokinetics, Pharmacodynamcis and Pharmacogenetics, University of Wisconsin Comprehensive Cancer Center, Madison, Wisconsin, USA). The patients' consent to participate was obtained in compliance with existing institutional review board requirements.

Sample collection

Ten milliliters of whole blood was collected from each patient on a return clinic visit after their hospitalization. The specimens were frozen and shipped on ice to the reference laboratory for genotyping.

Extraction of genomic DNA

The genomic DNA was extracted from the patients' white blood cells by standard methods and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), with yields of 9.6 and 16.8 μg for case 1 and case 2, respectively. The purified genomic DNA was stored at 4°C for pending analysis.

Analysis of single nucleotide polymorphisms

The genomic analysis of SNPs was carried out for both patients in the following genes: *ABCB1* (rs1045642, rs1128503, and rs2032582), *GSTP1* (rs1695), *MGMT* (rs12917, rs1803965, rs2282164, rs2308318, rs2308321, rs2308322, and rs2308327), and *NQO1* (rs1800566). PCR was used to amplify the regions of the genomic DNA containing the selected SNPs in the patient and control samples (genomic DNA from a normal blood sample and/or HT29 colon cancer cells). The PCR reaction mixture contained 7.5 pmol of forward and reverse primers, template DNA (3 μl), 2X PCR master mix, (15 μl; Promega, Madison, Wisconsin, USA) and nuclease-free water to a final volume of 30 μl. The primers (sequences available upon request) were designed using the Pyromark Assay Design 2.0 software (Qiagen, Valencia, California, USA) and synthesized by Integrated DNA Technologies (Coralville, Iowa, USA) with either of the forward or reverse primer of each set biotinylated at the 5′ ends. The PCR was performed in an MJ Research PTC-200 (Waltham) thermal cycler with the following settings: 95°C – 5 min; 55 cycles of 95°C – 30 s, 51–53°C – 30 s, 72°C – 1 min; 72°C for 5 min with storage at 4°C post cycle. To assess proper amplicon length, 5 μl of each PCR product was subjected to electrophoresis on a 2% agarose gel containing 0.4 μg/ml of ethidium bromide and compared with a pGEM DNA ladder (Promega).

The genotype of each SNP was determined by pyrosequencing analysis on a PSQ 96 MA pyrosequencer (Biotage; Uppsala, Sweden) using PyroGold reagents (Qiagen) following the manufacturer's protocol. To prepare the PCR product for pyrosequencing, 20 μl of the biotinylated PCR product was immobilized on streptavidin-coated sepharose beads (GE Healthcare, Piscataway, New Jersey, USA) by constant agitation for 10 min at ambient temperature in 10 mmol/l of Tris–HCl,

at pH 7.6 containing NaCl (2 mol/l), EDTA (1 mmol/l), and Tween 20 (0.1%). The beads containing the bound-biotinylated PCR product were then treated by sequential washes with 70% ethanol, NaOH (200 mmol/l), and Tris–acetate (10 mmol/l), at pH 7.6, resulting in single-stranded DNA bound to the sepharose beads. The DNA–sepharose beads were then transferred to a 96-well microplate with 40 µl per well of 20 mmol/l Tris-acetate, pH 7.6 containing magnesium acetate (2 mmol/l) and sequencing primer (21 pmol). Annealing of the sequencing primer to the biotinylated PCR product was then performed by incubation at 80°C for 2 min followed by cooling to ambient temperature. Run parameters were set up in the pyrosequencer's control software and data analysis was done using the PSQ 96 SNP 2.1 software (Biotage), which provided genotyping results on the basis of peak height measurements.

Assessment of O^6-methylguanine-DNA-methyltransferase promoter methylation status

To determine the extent of MGMT promoter methylation, the pyrosequencing method of Dunn *et al.* [16] was used with all primers and thermocycler settings identical to those reported in their paper. It should be noted that in addition to the genomic DNA from both patients, genomic DNA from six normal blood samples was included for comparison along with genomic DNA from PC3 and PPC1 prostate cancer cell lines that had been universally methylated by treatment with *Sss*I CpG methylase (New England Biolabs, Ipswich, Massachusetts, USA) according to the manufacturer's protocol. Bisulfite conversion of 500 ng of each genomic DNA sample was done before PCR using either the EZ DNA methylation gold kit (Zymo; Orange, California, USA) or the EpiTect Bisulfite Kit (Qiagen). The PCR reaction mixture was as described above but used a 2X GoTaq Hot Start colorless PCR master mix (Promega). Pyrosequencing was set up as described above and the methylation status of each of the 12 CpG sites within the MGMT promoter was analyzed using Pyro Q-CpG 1.0.9 software (Biotage).

Results

Genotyping analysis of genomic DNA for selected single-nucleotide polymorphisms

On the basis of a review of the literature, several candidate SNPs were selected for analysis as a consequence of their postulated roles in either the clinical response or toxicity of TMZ therapy [3,9,10,19–21,23–26]. The SNPs are located in specific genes that encode for the following enzymes: MGMT, MDR1 P-glycoprotein (also called as ABCB1), NQO1, and GSTP1.

The results of the genotyping analysis for the patients are listed in Table 1. It is notable that of the 12 SNPs assessed, non-wild-type variants were frequent (8/12 and 5/12 for cases 1 and 2, respectively) in contrast to the population frequencies. Non-wild-type variants were observed for one SNP in GSTP1 (rs1695), four in MGMT (rs12917, rs1803965, rs2308321, and rs2308327) and all three in ABCB1 (rs1045642, rs1128503, and rs2032582). In contrast, both patients were found to be of wild type for NQO1 (rs1800566) and for the remaining MGMT SNPs (rs2282164, rs2308318, and rs2308322), which, in the latter case, is consistent with the low prevalence of these SNPs in the Caucasian demographics (frequencies are given in Table 1) [27].

The two patients exhibited different genotype profiles for the selected SNPs. Although both patients were

Table 1 Summary of genotyping results of the selected SNPs[a]

SNP	Case 1	Case 2	Genotype with increased risk[b]	SNP type[c]	Frequency in Caucasian[d,e]
ABCB1					
rs1045642	**CT**	**CT**	–	Synonymous	CC: 0.15, CT: 0.63, TT: 0.22
rs1128503	**CT**	**CT**	–	Synonymous	CC: 0.35, CT: 0.52, TT: 0.13
rs2032582	**GT**	**GT**	–	missense (S893A)	GG: 0.32, GT: 0.56, TT: 0.12
GSTP1					
rs1695	**AG**	AA	AA	missense (I105V)	AA: 0.33, AG: 0.55, GG: 0.12
MGMT					
rs12917	**CT**	**CT**	–	missense (L84F)	CC: 0.78, CT: 0.20, TT: 0.02
rs1803965	**GA**	**GA**	–	Synonymous	GG: 0.85, GA: 0.14, AA: 0.02
rs2282164	CC	CC	–	missense (W65C)	CC 1.00, CG and GG are <0.001
rs2308318	GG	GG	–	missense (G160R)	GG 1.00, AG is 0.007 globally, AA: 0.00
rs2308321	**GG**	AA	AG or GG	missense (I143V)	AA: 0.65, AG: 0.35; GG: 0.007 globally
rs2308322	GG	GG	–	missense (P58S)	GG, 1.00, GA is 0.003 globally, AA 0.00
rs2308327	**AG**	AA	AG or GG	missense (K178R)	AA: 0.98, AG: 0.02, GG: 0.00
NQO1					
rs1800566	GG	GG	GG	missense (P187S)	GG: 0.60, AG: 0.37, AA: 0.03

ABCB1, ATB-binding cassette subfamily B member 1; GSTP1, glutathione S-transferases pi 1; MGMT, O^6-methylguanine-DNA-methyltransferase; NQO1, NAD(P)H dehydrogenase (quinone) family; SNPs, single nucleotide polymorphisms;

[a]Bold lettering denotes SNPs, which were found to be non-wild-type for the indicated patient.

[b]Listed genotypes have been shown to be associated with an increased risk of temozolomide-induced myelosuppression as indicated by Armstrong *et al.* [19].

[c]A synonymous SNP is one in which there is no amino acid change.

[d]Frequencies in caucasian from the HapMap CEU dataset, [27] whereas global frequencies are from the NIH Polymorphism Discovery Resource (NIHPDR) [28].

[e]The wild-type genotype appears first, followed by the heterozygote, and the variant.

heterozygous for all ABCB1 SNPs and for rs12917 and rs1803965 in MGMT, patient 1 was heterozygous for rs1695 in GSTP1 and rs2308327 in MGMT. This patient also exhibited the GG genotype for the MGMT SNP, rs2308321, which is noteworthy for its 0.7% global frequency [28].

Analysis of O^6-methylguanine-DNA-methyltransferase promoter methylation status

In addition to the potential of the aforementioned SNPs to predispose the patients to myelosuppression after TMZ administration, it was considered that epigenetic factors might also play a role. Of specific interest was the effect of epigenetic silencing of MGMT, which has been reported to occur by hypermethylation of the gene's promoter region at the CpG islands [12,13]. The methylation status of the *MGMT* gene promoter region was assessed for both patients using the pyrosequencing method described by Dunn *et al*. [16] The patients were found to have an average of 1.43 ± 0.82 and 1.48 ± 0.98% methylation (case 1 and 2, respectively) across all of the 12 CpG sites within the MGMT promoter assayed. This was in agreement with the methylation status of the normal blood control samples, which featured an average of 1.98 ± 0.68% methylation and was markedly different from positive control samples (genomic DNA that was universally methylated by treatment with *Sss*I CpG methylase) that exhibited an average of 92.28 ± 10.3% methylation. As the average methylation for both patients was very low and well within the standard deviation of the normal controls, it was concluded that the MGMT promoter regions were unmethylated, and did not differ from that of normal blood samples.

Discussion

Earlier studies have investigated the roles of several genes in relation to the clinical response and/or toxicity of TMZ [6,7,13,14,16,18,19,21]. Of the various genes that have been postulated to modulate the activity of TMZ *in vivo*, arguably the most important is *MGMT* [7]. The *MGMT* gene is responsible for the repair of DNA adducts formed by several alkylating agents, including TMZ [11,13]. The activity of methyltransferase was modulated in tumor cells by silencing of the *MGMT* gene expression by promoter methylation [12,13]. The patients with a methylated promoter have decreased MGMT-mediated DNA repair, a feature that could be relevant for TMZ-induced toxicity. Our finding of unmethylated MGMT promoter in the germ line of both patients is consistent with that of Nagane *et al*. [18]. These results suggest that decreased activity of MGMT by silencing of the *MGMT* gene is not the mechanism causing severe TMZ-induced myelosuppression in these two patients or those studied by Nagane *et al*.

In addition to the role of promoter methylation status of *MGMT*, several polymorphisms of this gene have been investigated for their influence on function and expression of the gene product [9,10]. Although the effects of many MGMT polymorphisms have not been elucidated, some have been shown to affect enzyme activity [10], and affect the levels of enzymatic activity in peripheral blood mononuclear cells [25]. This is important as functional polymorphisms within the *MGMT* gene even in the absence of promoter methylation may decrease the ability of cells to repair themselves and potentially increase the risk of TMZ-induced myelosuppression.

In this regard, the genotyping results for MGMT are interesting. Both patients developed severe TMZ-induced myelosuppression despite different genetic profiles for MGMT polymorphisms. Although both patients were found to be heterozygous for the synonymous rs1803965 SNP and the missense L84F (rs12917) polymorphism, patient 1 was also non-wild-type for the missense I143V and K178R polymorphisms (rs2308321 and rs2308327, respectively). Of the polymorphisms common between the two patients, the missense L84F (rs12917) polymorphism has not been shown to affect function of MGMT, although a putative effect of this polymorphism on MGMT expression levels has not been ruled out [10]. In contrast, the role of the synonymous rs1803965 polymorphism is essentially unknown [9], but has been postulated to alter the extent of gene methylation [24], a premise that is not supported by our analysis of the MGMT promoter region methylation status. Therefore, it is possible that this MGMT SNP (rs1803965) may play another functional role in the TMZ-induced myelosuppression observed for these patients.

Two polymorphisms that are unique to patient 1 (missense I143V and K178R polymorphisms; rs2308321 and rs2308327, respectively) have recently been shown to correlate with an increased risk of severe TMZ-induced myelosuppression. Specifically, patients with either the GG or GA variants exhibited an overall risk for myelosuppression of 2.32 and 2.40 (rs2308321 and rs2308327, respectively) compared with the wild type [19]. This is consistent with our findings for patient 1 and may explain the more severe TMZ-induced myelosuppression experienced by this patient.

Armstrong *et al*. [19] also described SNPs within other genes that were associated with a decreased risk of myelosuppression after the administration of TMZ. Specifically, it was reported that patients who were non-wild-type variants for both NQO1 (rs1800566) and GSTP1 (rs1695) exhibited an overall risk of 0.30 and 0.28 for severe myelotoxicity versus wild type. *NQO1* gene is a member of the NAD(P)H dehydrogenase (quinone) family and encodes a cytoplasmic 2-electron reductase. Glutathione S-transferases are a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. Therefore, both NQO1 and GSTP1 in their normal state should be

protective against myelosuppression induced by TMZ, which works by DNA alkylation. The genotyping results for the patients described in this study indicate that both were wild type for rs1800566 in NQO1 but patient 1 was heterozygous for rs1695 in GSTP1. These findings suggest that patient 2 did not exhibit any of the 'protective' polymorphisms, which are associated with a decreased risk of myelotoxicity. Interestingly, the fact that patient 1 was a non-wild-type variant for GSTP1 implies that the putative protective effect of this polymorphism was not able to counteract the risk factors, which led to this patient's severe myelotoxicity. Perhaps, the effects of a protective polymorphism, such as GSTP1 (rs1695), can be overcome by effects of MGMT SNP (rs1803965). It is possible that the inheritance of a SNP that predisposes to toxicity because of inability to catabolize a drug might usually 'outscore' a protective SNP. This could be an important consideration when identifying patients at risk of toxicity.

Although it remains to be determined that TMZ is a substrate of the MDR1 P-glycoprotein efflux pump, it has been recently reported that the genotype of a specific ABCB1 polymorphism, which encoded for the enzyme, was predictive for clinical benefit from TMZ therapy [21]. The wild-type CC genotype of the ABCB1 SNP, rs1128503, was associated with an increased overall survival in patients with GBM treated with TMZ, implying that the wild-type genotype might be associated with reduced TMZ efflux from cancer cells. The same effect in myeloid cells could result in an increased risk of myelosuppression. Both our patients had CT genotype at this location and not CC. Therefore, the activity of the P-glycoprotein efflux pump does not explain the observed TMZ-induced myelosuppression in these cases.

Moreover, it has also been shown that in patients with acute myeloid leukemia treated with daunorubicin and cytarabine, a better outcome was observed for those who were heterozygous for the rs1045642 polymorphism in ABCB1 [23]. Apparently both the CC genotype of rs1128503 and the heterozygous genotype of rs1045642 resulted in decreased activity of the MDR1 P-glycoprotein: an observation that is confusing in light of the strong linkage disequilibrium between the ABCB1 SNPs [20]. The genotyping results of this study indicate that they were heterozygous for all the ABCB1 polymorphisms. Although it would be tempting to conclude that the latter polymorphism (rs1045642) is more relevant, the actual effect of these polymorphisms is equivocal at best. More insight into the role of TMZ as a P-glycoprotein substrate and into the clinical relevance of the ABCB1 polymorphisms is required before the relationship between these SNPs and the risk of severe myelotoxicity can be determined.

In conclusion, these data suggest that the risk of severe TMZ-induced myelosuppression is not solely dependent on a single polymorphism or the silencing of a specific gene. Further research is required to better describe the individual and collective impact of each polymorphism on the development of severe TMZ-induced myelosuppression. To determine whether genotyping analysis is a valuable tool to minimize this risk, studies of larger patient populations administered TMZ as standard adjuvant treatment of GBM are required. This study could potentially result in the development of 'susceptibility SNP panel', an effort that we are currently pursuing.

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