A *MDR1 (ABCB1)* gene single nucleotide polymorphism predicts outcome of temozolomide treatment in glioblastoma patients

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**Background:** Some patients with glioblastoma multiform do not respond to temozolomide even though they have aberrant promoter methylation of the DNA repair enzyme O6-methylguanine methyltransferase (MGMT). This suggests that additional factors hamper temozolomide cytotoxicity. We aimed to confirm first that temozolomide is a target for the multidrug resistance transporter MDR1/ABCB1 and second to investigate whether genetic variants of the *MDR1* gene are associated with the survival of glioblastoma patients treated with temozolomide.

**Materials and methods:** Temozolomide-mediated cytotoxicity was determined by the colorimetric methyl-thiazol-tetrazolium assay in MDR-expressing and MDR-nonexpressing cell lines. Genotypes of three single nucleotide polymorphisms (SNPs) of the *MDR1* gene (C1236T, G2677T, and C3435T), *MDR1* mRNA expression levels, and the *MGMT* promoter methylation status were analyzed in 112 glioblastoma patients who had been treated either by surgery plus radiotherapy alone or by additional temozolomide chemotherapy.

**Results:** In vitro analysis revealed that temozolomide-mediated cytotoxicity is dependent on MDR1 expression. Multivariate analysis of *MDR1* genotypes showed that the C/C variant of the exon12 C1236T SNP is predictive for survival of patients treated with temozolomide. This effect was independent of the *MGMT* methylation status. Patients with the C/C genotype had a 2-year overall survival of 37% compared with 8% and 10% for patients with C/T and T/T genotypes, respectively (*P* = 0.02). No influence was seen in the group of patients with radiotherapy only.

**Conclusion:** The genotype of the *MDR1* exon12 C1236T SNP is a novel independent predictive factor for outcome of temozolomide treatment in glioblastoma patients.

**Key words:** glioblastoma, MDR1, MGMT, polymorphism, temozolomide

**introduction**

Glioblastoma multiform (astrocytoma World Health Organization grade IV) is the most frequent and most malignant primary brain tumor in adults, causing ~13 000 deaths per year in the United States. The median survival is still in the range of 12 months in spite of multimodal aggressive treatment, comprising surgical resection, local radiotherapy, and systemic chemotherapy [1]. Recently, temozolomide has been introduced into standard care [2]. Temozolomide is a DNA-methylating agent with a broad-spectrum antitumor activity, the ability to cross the blood–brain barrier, a good safety profile with nonoverlapping toxic effects, an oral formulation, and the ability to overcome resistance to nitrosoureas [3]. It is effective in glioblastoma treatment as shown for recurrent glioblastoma first [4, 5] and, recently, in a prospective randomized trial conducted by the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) Clinical Trial Group (CTG), for newly diagnosed glioblastoma when administered concomitantly with and as an adjuvant after radiotherapy [6]. The DNA damage caused by methylating agents like temozolomide is reversed by the ubiquitous repair enzyme O6-methylguanine methyltransferase (MGMT) [7, 8]. The *MGMT* gene can be silenced epigenetically by hypermethylation of its promoter [9]. Transcriptional silencing of the *MGMT* gene by promoter hypermethylation is seen in ~50% of glioblastomas and has been linked to a better response to alkylating chemotherapy and longer survival in glioblastoma patients [10]. Therefore, *MGMT* methylation status represents the first molecular marker of prognostic and predictive significance for glioblastoma. However, results still need to be confirmed in a prospective randomized trial. Moreover, *MGMT* promoter methylation status is unlikely to be the only
molecular marker and mechanism that affects survival outcome for patients with glioblastoma.

Another mechanism mediating chemoresistance is the expression of efflux pump proteins that selectively transport substances out of cells. The multidrug resistance gene (MDR1/ABCB1, OMIM *171050) encodes for a P-glycoprotein (P-gp), which is a well-known drug efflux pump whose overexpression has been primarily observed in human cancer that is resistant to chemotherapy [11], particularly to drugs such as vincristine, etoposide, and doxorubicin [12]. Whether temozolomide is also a target for P-gp-mediated chemoresistance has not been well documented [13]. MDR1 has been found to be expressed in the majority of brain tumors, including glioblastomas [14]. The effectiveness of the P-gp efflux pump may be influenced in several ways: an altered ability of this P-gp to recognize target substrates, by other nontarget substances inhibiting the pump transporter and by genetic variants having a functional impact on MDR1 transcription [15, 16]. Among those, the three single nucleotide polymorphisms (SNPs) C1236T, G2677T*, and C3435T have been well characterized for their functional effect on disease development and drug response in various populations (reviewed by Pauli-Magnus and Kroetz [17]). In a previous study, we confirmed that those SNPs of the MDR1 gene had influence on treatment outcome in acute myeloid leukemia patients [18]. Therefore, in the present study, we aimed to confirm first that temozolomide is also a target for the P-gp efflux pump MDR1 and second to investigate whether genetic variants of the MDR1 gene are associated with the survival of glioblastoma patients receiving temozolomide treatment.

materials and methods

in vitro cytotoxicity

In vitro cytotoxicity was measured using the colorimetric methyl-thiazol-tetrazolium (MTT) assay. Cells from the chemosensitive parental MDR1-negative chronic myeloid leukemia cell line K562 and the strongly MDR1-positive derived line K562-VP16 were cultured with RPMI medium and only for K562-VP16 with 5 μM etoposide [19]. From the parental and resistant line, 1 × 10⁶ cells were incubated with 10 μg/ml temozolomide, 1 μM cyclosporine A, 10 μg/ml temozolomide plus 1 μM cyclosporine A and without any drugs as a control. Experiments were done eight-fold for 48 h in wells of microculture plates at 37°C in humidified air containing 5% CO₂. After 48 h, the MTT assay was carried out as previously described [19]. Mean values and standard deviations of six independent experiments were determined.

study population

The tumor database of the Department of Neurosurgery, University Hospital in Dresden, was used to identify adult patients who had been operated for supratentorial glioblastoma during the years 1998–2006, had adjuvant locoregional radiotherapy, and either had no adjuvant chemotherapy or had temozolomide concomitantly and/or adjuvant to radiotherapy. All patients had given informed written consent for molecular analyses of tumor tissue, and the study had been approved by the local ethics committee.

Histopathology was carried out at the Department of Neuropathology, Dresden University Hospital, and only cases with an unequivocal diagnosis of glioblastoma were selected. For patient characteristics see Table 1.

Table 1. Patient characteristics

<table>
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<tr>
<th></th>
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<td>Daily temozolomide dosage/cycle (mg/m²)</td>
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DNA and mRNA isolation

Tumor tissues were taken intraoperatively and were snap frozen at −80°C. Frozen tumor tissue was sectioned using a cryotome (CryoStat Jung CM 1800, Leica). To assure a tumor cell content of at least 80% for nucleic acid extraction, control slides stained with hematoxylin and eosin were examined by the local neuropathologist. For DNA isolation, the QiAmp DNA Mini Kit 50 (Qiagen, Hilden, Germany) and for RNA isolation, the Invitrogen Spin Tissue RNA Mini Kit 250 (Invitrogen, Berlin, Germany) were used according to the manufacturers’ instructions. The quantity and purity of RNA were estimated spectrophotometrically and their integrity was checked by electrophoresis in 1% agarose gel. RNA was reverse transcribed using the Omniscript RT Kit (Qiagen).

PCR of polymorphic sites of the MDR1 gene

The most frequent polymorphic sites of the MDR1 gene comprising the exon12 C1236T (rs1128503), exon21 G2677T* (rs2032582), and exon26 C3435T (rs1045642) SNPs were amplified by PCR. SNP positions refer to the MDR1 cDNA sequence [20], with the first base of the ATG start codon set to 1. PCR was carried out on genomic DNA using the previously described primer molecules [18]. Primer sequences and PCR conditions are described primer molecules [18], Primer sequences and PCR conditions are provided on request. PCR products were analyzed on standard 1.5% agarose gels stained with ethidium bromide (0.5 μg/ml).

SSCP analysis and sequencing

For SSCP analysis, the PhastGel system (Amersham Pharmacia Biotech, Freiburg, Germany) was used as previously described [18]. Primer pairs covering the described sequence strings additionally may amplify polymorphic loci of exon21 and 26. However, the frequencies of these polymorphisms are low. The observed frequency for cDNA2677 G/A and T/A genotypes together is 3.8% [21] and for the heterozygous genotype of the C3396T SNP is 0.5% [15]. We therefore did not consider those changes in our survey and excluded patients with SSCP ‘extra bands’.

For confirmation of SSCP results, bands from representative samples of each genotype of all three SNPs were cut out, eluted, and prepared for sequencing by an ABI 3730x (Applied Biosystems) using the same primers as for the detection of the polymorphic sites of the MDR1 gene (see above).
real-time PCR
To determine the amount of MDR1 mRNA expression, real-time PCR was carried out using primers and probes according to Schiedmeier et al. [22]. GAPDH was used as positive control. Primers and probes were purchased from TibMolBiol (TibMolBiol, Berlin, Germany). PCR reactions were run in triplicate. Amplification conditions were 2 min preincubation time at 95°C, 10 min at 95°C enzyme activation and 50 cycles at 95°C for 15 s denaturation and 60°C for 1 min annealing and extension. Quantitative results were assessed by determination of the critical threshold cycle (Ct) as described previously [18, 23].

immunohistochemistry
Samples from 10 tumors each exhibiting overexpressed and low amounts of mRNA in real-time PCR, respectively, were analyzed. Frozen tissue was cut into 8-μm slices and fixed by acetone. The Streptavidin-fluorescein isothiocyanate technique was used for immunohistochemistry applying the primary goat polyclonal antibody Mdr C-19:sc-1517 (Santa Cruz Biotehnologies, Inc., Santa Cruz, CA) raised against a peptide mapping at the C-terminus of Mdr-1 of human origin and the secondary anti-goat biotin-labeled antibody. For controls, K562 and K562-VP16 cell lines (see above) were used. Tissue spins from those cells were prepared by cytocentrifuge preparations of 1×10⁶ cells (60 g, 7 min, Cytospin Shandon, France) in PBS. Spins were air dried for 2 h and fixed in acetone for 10 min at 4°C. Slides were visualized using a spectral microscope with AxionVision® software (Zeiss, Göttingen, Germany).

MGMT promoter methylation analysis
MGMT promoter methylation was analyzed by methylation-specific PCR as reported before [24]. The primer sequences used to detect methylated MGMT promoter sequences were 5′-gtttgtaaggttgttgttcgg-3′ and 5′- cactgccagaaaaaactcg-3′ (corresponding to nucleotides 46912-47033, GenBank accession no. AL355531, fragment size: 122 bp). The primer sequences used to detect unmethylated MGMT promoter sequences were 5′-tttttaagtttttgttttgat-3′ and 5′-tcaccatctccccaaaaacctcca-3′ (corresponding to nucleotides 46909-47037, fragment size: 129 bp). As positive control sample, we used the A172 glioma cell line, which has a completely methylated MGMT promoter. Genomic DNA from a meningioma served as an unmethylated control sample.

statistical analyses
The Mann–Whitney U-test was used for comparison of the results. Differences in clinical or laboratory parameters were compared between the in vitro MTT assays or between the various SNPs of the MDR1 gene using the Mann–Whitney test for continuous and the chi-square test for categorical variables. Overall survival (OS) was measured from the date of diagnosis until death (failure) or last follow-up date (censoring). The Kaplan–Meier method was used to estimate OS probabilities, which were compared between different groups using the log-rank test. The Cox model was applied to identify independent prognostic variables for survival. Only patients with complete data for all variables considered were included in multiple models. A stepwise backward-selection procedure was carried out in order to determine the final model. The significance level was 0.05. To provide quantitative information on the relevance of results, 95% confidence intervals (CIs) of odds ratios and hazard ratios (HRs) were computed. All the above mentioned statistical analyses were carried out with the software package of SPSS Version 12.0.1 (SPSS Inc., Chicago, IL) and the S-PLUS software package version 6.2 (Insightful AG, Reinach, Switzerland).

The deviation from the Hardy–Weinberg equilibrium was tested using the chi-square test. For pair wise linkage disequilibrium between the genetic markers, three estimators, D, D', and r were computed. These analyses were carried out using the free statistical computing environment R (Version 2.3.1, http://www.r-project.org/) and its library genetics for genetic analyses.

results
in vitro cytotoxicity of temozolomide
The results of the MTT cytotoxicity assays are presented in Figure 1. In the chemosensitive MDR1-negative parental cell line K562 10 μg/ml temozolomide resulted in pronounced cell death with only 47.1% surviving 48 h compared with the control. In contrast, in the highly MDR1-expressing resistant subline K562-VP16, cell death was significantly lower after exposure to temozolomide with 73.4% surviving 48 h (P = 0.002). Addition of a nontoxic dose of the MDR1-modulator cyclosporine A (1 μM) to temozolomide resulted in a trend towards restoration of chemosensitivity in the resistant MDR1-expressing cell line. However, this difference in survival was not statistically significant. As both cell lines were methylated at the MGMT promoter site (data not shown), these results indicate that MDR1 participates in the resistance of tumor cells to temozolomide.

genotype frequencies
MDR1 genotypes of exon12 C1236T, exon21 G2677T, and exon26 C3435T SNPs were analyzed by SSCP (Figure 2) in all 112 glioblastoma patients. Genotype frequencies are depicted in Table 2 and were in accordance with those predicted by the Hardy–Weinberg equilibrium (P = 0.33, 1.0, and 0.85 for exon12, 21, and 26 SNPs, respectively).

All three examined gene polymorphisms were in a linkage disequilibrium to each other (P < 0.0001 for each possible pair). This is reflected by the distribution of the detected
combinations of the allelic variants (D’ 0.85, 0.68, and 0.76 for exon21/12, 26/12, and 26/21 combination, respectively).

**MDR1 mRNA and P-gp expression**

mRNA with sufficient quantity and integrity was available from 99 tumor samples. The median MDR1 mRNA expression was 5119 copies with a broad interindividual range of 1–165 628 copies. MDR1 mRNA expression correlated with P-gp expression as shown in 20 tumor tissue samples by immunohistochemistry (data not shown). Interestingly, the median MDR1 expression was higher in patients with the heterozygous variant of the exon12 and 21 SNPs and lower in patients with the heterozygous state of exon26 SNP compared with all other patients (Table 2). However, these differences were not statistically significant.

**MGMT promoter methylation**

Methylation of the MGMT gene promoter was found in 37 (34%) of 110 examined patients. Methylation status was hardly different between the two treatment groups (32% for patients receiving radiotherapy only and 35% for patients receiving radiotherapy plus temozolomide; P = 0.45). Furthermore, no significant difference of the MGMT methylation status was detected between the examined SNPs of the MDR1 gene (Table 2).

**effect of MDR1 gene variants and MGMT promoter methylation on treatment outcome**

The median OS was 11.0 (8.1–13.8) months for all 112 glioblastoma patients. Median survival was 8.5 months for patients receiving radiation therapy only (n = 48) and 15.8 months for patients (n = 64) receiving temozolomide in addition to radiation therapy (log-rank test: P < 0.001, data not shown). Potentially prognostic factors included in a multiple Cox model were gender, age, Karnofsky performance score, postoperative treatment group, MGMT methylation status and MDR1 genotypes of exon12, 21, and 26 SNPs (n = 108 patients with complete data). As MDR1 expression had no influence on survival in the univariate analysis and data were available only for 99 patients, this factor was not included. Postoperative treatment comprising radiotherapy plus temozolomide was protective, resulting in a HR of 0.33 (0.21–0.52) (P < 0.001) (Table 3).

In the group of patients receiving temozolomide in addition to radiotherapy, the MGMT methylation status was predictive of treatment outcome. Patients with methylated MGMT (n = 22) survived longer than patients with an unmethylated MGMT promoter (n = 41). The 2-year OS rates were 27% versus 13%, respectively (log-rank test: P = 0.01, data not shown). In addition, patients with the C/C allele of the exon12 C1236T SNP had better 2-year OS compared with patients with the heterozygous variant (n = 28) (37% versus 8%; log-rank test; P = 0.02). Patients with the less frequent T/T variant (n = 11) had a 2-year OS of 10%. Due to the low number of patients with this variant no valid statistical conclusion in comparison with patients harboring the other two variants was applicable (Figure 3A). In the Cox model including above candidate variables (n = 61 patients receiving temozolomide with complete data), the MDR1 genotype of the exon12 C1236T SNP was an independent predictive factor for outcome of temozolomide therapy (P = 0.02). The HR for death of patients with the C/C allele was 0.25 (95% CI 0.10–0.65) compared with patients with the heterozygous variant (P = 0.004) (Table 3).

No influence of the exon12 C1236T SNP genotypes on survival was seen in the group of patients with radiation therapy only (n = 48) (Figure 3B).

**discussion**

In the present study, we have shown that the MDR1 encoded P-gp efflux pump is one of those factors, which are involved in resistance to temozolomide-mediated cytotoxicity. In vitro survival of MDR1 expressing K562 cells was significantly higher than in the MDR1-negative parental cell line on incubating the cells with temozolomide. Both cell lines had a methylated MGMT promoter. Jelinek et al. [13] transfected K562 cells with a retroviral vector containing MDR1 and MGMT and correlated temozolomide resistance of the cells with MGMT expression. However, they did not test the influence of MDR1.
expression alone on temozolomide resistance. Significant MDRI P-gp expression was found in both low- and high-grade gliomas, suggesting an intrinsic resistance to anticancer drugs [14]. Particularly, endothelial cells within gliomas stained positive for MDRI P-gp [25, 26] supporting the idea that chemoresistance may be caused not only by P-gp expression in cancer cells but also by its expression in the endothelial cells of brain tumor capillaries. Thus, as it is known that genetic cancer cells but also by its expression in the endothelial cells of chemoresistance may be caused not only by P-gp expression in cancer cells but also by its expression in the endothelial cells of brain tumor capillaries. Thus, as it is known that genetic polymorphisms of the MDRI gene may affect the expression and function of the P-gp efflux pump [15, 18, 27], we examined three polymorphisms of the MDRI gene in exon12, 21, and 26 in the tumor specimens of 112 patients with glioblastoma. The genotype frequencies of those SNPs in our study population was comparable to the distribution found in the normal Caucasian population [21] and is in line with a recently published survey of Miller et al. [28]. They found no significant association of the exon26 C3435T polymorphism and the occurrence of glioma.

In our analysis, the exon12 C1236T polymorphism of the MDRI gene turned out to be an independent predictor of the outcome of temozolomide treatment in glioblastoma patients. Patients treated with radiotherapy plus temozolomide harboring the more frequent C/C allele had superior 2-year OS compared with patients with the heterozygous variant. This effect was independent of well-known prognostic factors like age and Karnofsky performance score and particularly the MGMT methylation status and was not seen in the group of patients having received radiotherapy only, indicating a specific role of MDRI in the response to temozolomide in glioblastoma patients. This is further supported by a lower MDRI1 expression in the tumor samples of patients harboring the C/C allele at the examined exon12 locus compared with heterozygous patients. It also accords with our previous study of patients with acute myeloid leukemia [18], where allelic variants of the MDRI gene including the exon12 C1236T SNP were found to influence therapy outcome. However, the way in which the exon12 polymorphism influences MDRI1 gene expression and protein function is not known yet. One explanation might be functional linkage to polymorphic positions at regulatory sites of the MDRI promoter. This hypothesis is supported by the linkage disequilibrium detected between the exon12, 21 and 26 SNPs, indicating that linkage of polymorphisms in the MDRI gene is a common phenomenon, as shown for healthy subjects [27] and in patients with acute myeloid leukemia [18].

Kimchi-Safra et al. [29] have shown that substrate specificity of the encoded P-gp is altered by a synonymous SNP (C3435T) which is part of the haplotype comprising the exon12, 21, and 26 SNPs, suggesting that an altered affinity of P-gp for temozolomide might be a reason for the survival differences after temozolomide therapy in our study.

Nevertheless, we cannot rule out the possibility that the C/C genotype of the exon12 C1236T SNP might be an epiphenomenon accompanying other resistance mechanisms, like mismatch repair (MMR) pathway deficiency or microsatellite instability (MSI). However, in glioblastoma, MSI and underlying MMR gene mutation were found only in subgroups of patients like long-term survivors or particularly young patients, but infrequently in common cases [30].

In conclusion, the outcome of temozolomide treatment in glioblastoma patients can be predicted by evaluating the genotype of the MDRI exon12 C1236T SNP. This finding seems to be independent of the MGMT methylation status. However, our data have to be verified in a prospective trial.
The influence of the C/C allele on survival was found in this group. (A) Patients receiving postoperative treatment with radiotherapy + temozolomide (n = 64) (C/C genotype versus C/T genotype: log-rank test P = 0.02). (B) Patients receiving postoperative radiotherapy only (n = 48). No influence of the C/C allele on survival was found in this group.

where the MDR1 exon12 C1236T genotype is analyzed in combination with the MGMT promoter methylation status.

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**references**


